ATTACHMENT IV

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Enhancement by non-mutagenic pesticides of GST-P positive hepatic foci development initiated with diethylnitrosamine in the rat

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Summary

The potential hepatocarcinogenicity of seven pesticides was examined using a rapid bioassay based on the induction of glutathione *S*-transferase placental form positive foci in the rat liver. Rats were initially injected with diethylnitrosamine and two weeks later were fed on diet supplemented with one of the pesticides for 6 weeks and then killed; all rats were subjected to a partial hepatectomy at week 3. Positive results were seen with chlorobenzilate (2000 ppm), vinclozolin (2000 ppm), malathion (4000 ppm), tecnazene (2000 ppm) and isoproturon (2000 ppm). S,S,S-tributylphosphorotrithioate (DEF, 200 ppm) and dicloran (2000 ppm) were negative in both number and area analyses. Although chlorobenzilate is carcinogenic in mice, malathion and vinclozolin have been reported as non-carcinogens in both rats and mice. Since the present system is based on the two-stage carcinogenesis hypothesis, it is possible that the chemicals showing positive results in this system possess at least tumor-promoting activity in the rat liver. This is very significant, as most carcinogens show tumor-promoting activity in their target organs.

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Introduction

Investigation of possible environmentally derived influences in animal experiments has established that a vast array of compounds are capable of playing a role in tumorigenesis; and detection and appropriate regulation of these compounds are of prime importance for the prevention of neoplasia in humans. Agricultural chemicals are recognized as possible environmental toxic or carcinogenic agents [1,2]. Not only workers in industry and agriculture but also the general population may be exposed to those chemicals from foods. Evaluation of potential carcinogenic or tumor-modifying activities of agricultural chemicals is therefore clearly very important.

In our laboratory a medium-term bioassay system of only 8 weeks' duration utilizing rat liver glutathione *S*-transferase placental form (GST-P) positive foci as end-point markers has been introduced to bridge a long-term carcinogenicity bioassay and in vitro screening tests; the reliability of the system is reviewed elsewhere [3–6]. Using this system, we have examined possible carcinogenicity of a variety of pesticides. The criteria used to select the compounds were (i) evidence of toxicity as described in the literature, (ii) widespread use, (iii)

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chemical structure suggestive of carcinogenic potential, or (iv) structural similarity to other suspected agents [7].

Materials and Methods

Chemicals

Diethylnitrosamine (DEN) was obtained from Tokyo Chemical Company, Tokyo. The pesticides used were either obtained from commercial sources or received as gifts from the following: vinclozolin from BASF India Ltd., Bombay; malathion from Cheminova, Lemvig, Denmark; S,S,S-tributylphosphorotrithioate (DEF) a gift from Dr. Cabral; chlorobenzilate from Nippon Kayaku Co. Ltd., Tokyo; tecnazene from Aldrich Chemical Company, Milwaukee, Wisconsin; isoproturon from Hoechst AG, Frankfurt; and dicloran from Wako Pure Chemical Industry, Ltd., Osaka.

Animals and treatments

A total of 194 male F344 rats (Charles River Japan Inc., Atsugi), 6 weeks old at commencement, were maintained on basal diet (Oriental M, Oriental Yeast Company, Tokyo) ad libitum and housed in plastic cages in an air-conditioned room at 24 ± 2°C and 55 ± 5% humidity with a 12 h/12 h light-dark cycle.

The animals were divided into three groups, as shown in Fig. 1. Group 1 was given a single i.p. injection of DEN (200 mg/kg body weight) dissolved in saline to initiate hepatocarcinogenesis and, after a 2 week recovery period on basal diet, received one of the test compounds for the next weeks. Animals were subjected to two-thirds partial hepatectomy (PH) at week 3 to maximize any interaction between cell proliferation and the effects of the compounds tested. Group 2 was given DEN and PH in the same manner as group 1 without any test compound. Group 3 rats were injected with saline instead of DEN solution and then subjected to test compounds and PH. Test compounds were given in the basal diet at the following concentrations: vinclozolin, 2000 ppm; malathion, 4000 ppm; DEF, 200 ppm; chlorobenzilate, 2000 ppm; tecnazene, 2000 ppm; isoproturon, 2000 ppm; and dicloran, 2000 ppm. The doses of test compounds were chosen on the basis of preliminary long-term tests or toxicity data, if available [8–14].

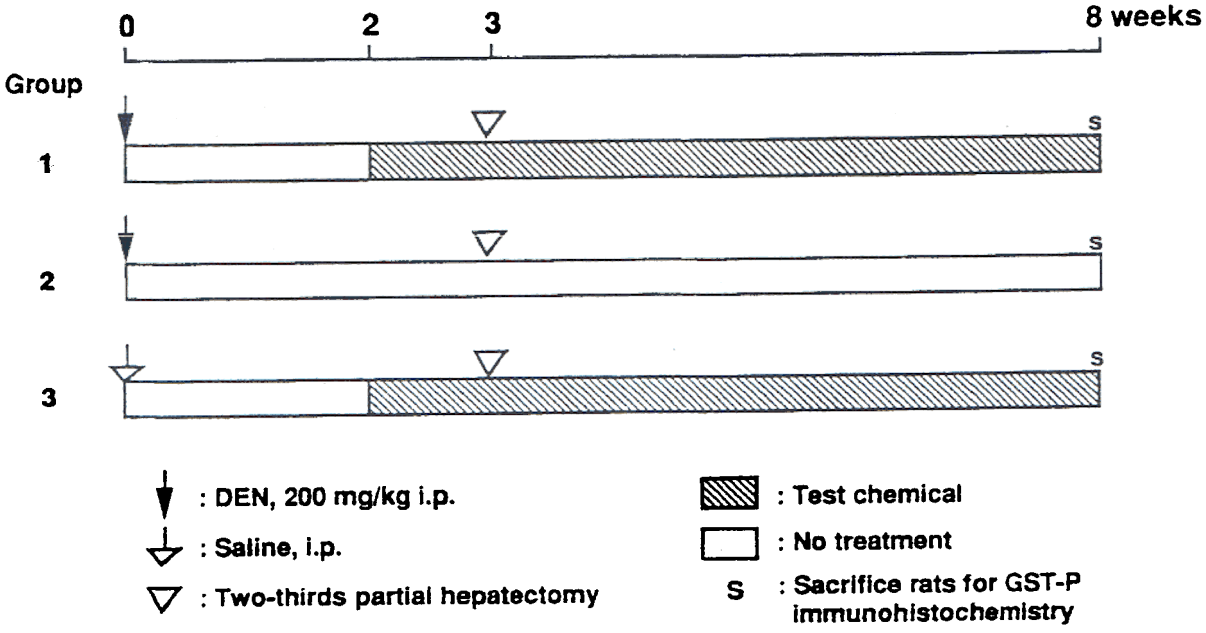

Fig. 1. Experimental protocol.

All surviving animals were sacrificed at week 8, livers were immediately excised, and sections 2–3 mm thick were cut with a razor blade. Three slices, one each from the right posterior, caudate and right anterior lobes, were fixed in ice-cold acetone for immunohistochemical examination of GST-P.

Tissue processing and GST-P positive foci analysis

GST-P immunohistochemistry was performed, as previously described [6,15]. The numbers and the areas of GST-P positive foci of more than 0.2 mm in diameter and the total areas of the liver sections examined were measured using a video image processor (VIP-21C, Olympus Company, Tokyo), as previously reported [16]. The results were assessed by comparing the values of foci between group 1 (DEN test compounds) and group 2 (DEN alone). Group 3 animals served to assay the potential of the test chemicals to induce GST-P positive foci without prior DEN.

Statistical analysis

Statistical analysis was carried out using Student's *t*-test and Welch's *t*-test in combination with the *F*-test for means [17]. A positive result was considered to be obtained when there was an increase at $P < 0.05$ in either number or area of foci between groups 1 and 2.

Results

The numbers of survivors in the experimental groups is shown in Table I. Overall mortality following PH was 2.6%, and no treatment-related mortality was seen. Final body and liver weights and data for food consumption during the period of feeding of the test chemical are summarized in Table I. Body weights as compared with the DEN-only group were significantly decreased in the vinclozolin and DEF groups. Absolute liver weights were increased in the malathion, chlorobenzilate, tecnazene, isoproturon and dicloran-treated groups, and relative liver weight (to body weight) was increased in all groups. In the non-initiated groups, body weight was slightly higher than those in the initiated groups, and the relative liver weight was also increased similarly to the initiated groups.

Numbers and areas of GST-P positive foci per unit area of liver section after treatment with and

Table I. Final body and liver weights of rats.

Group Treatment	No. of rats	Body weight (g)	Liver weight	
				Liver/body (%)
1. DEN				
Vinclozolin	16	254 ± 15[c]	8.8 ± 0.6	3.49 ± 0.09[a]
Malathion	16	260 ± 17	9.9 ± 1.2[a]	3.80 ± 0.45[a]
DEF	16	242 ± 15[a]	8.1 ± 0.7	3.35 ± 0.22[b]
Chlorobenzilate	16	266 ± 9	10.9 ± 0.6[a]	4.09 ± 0.15[a]
Tecnazene	14	273 ± 10	11.5 ± 0.7[a]	4.19 ± 0.14[a]
Isoproturon	15	270 ± 13	10.6 ± 0.8[a]	3.90 ± 0.18[a]
Dicloran	14	271 ± 14	12.5 ± 0.9[a]	4.62 ± 0.28[a]
2. DEN alone	15	267 ± 19	8.4 ± 1.0	3.13 ± 0.16
3. Vinclozolin	10	268 ± 16	9.0 ± 0.8	3.36 ± 0.15
Malathion	9	286 ± 10	10.8 ± 0.6	3.77 ± 0.14
DEF	10	254 ± 9	8.3 ± 0.6	3.28 ± 0.22
Chlorobenzilate	8	284 ± 20	12.7 ± 1.1	4.47 ± 0.22
Tecnazene	10	282 ± 15	12.1 ± 0.5	4.30 ± 0.21
Isoproturon	10	288 ± 12	10.9 ± 0.5	3.79 ± 0.22
Dicloran	10	274 ± 15	12.1 ± 0.6	4.42 ± 0.16

Significantly different from group 2 at [a]$P < 0.001$, [b]$P < 0.01$ or [c]$P < 0.05$, respectively.

Table II. Results of GST-P positive foci analysis.

Group	Treatment	No. of rats	GST-P positive foci	
				Area (mm^2/cm^2)
1. DEN				
	Vinclozolin	16	16.08 ± 4.62[a]	1.48 ± 0.49[a]
	Malathion	16	12.81 ± 4.07[b]	1.11 ± 0.49[c]
	DEF	16	6.92 ± 2.43	0.48 ± 0.20[c]
	Chlorobenzilate	16	13.89 ± 3.29[a]	1.27 ± 0.39[a]
	Tecnazene	14	12.70 ± 2.46[a]	1.04 ± 0.27[b]
	Isoproturon	15	18.76 ± 2.94[a]	1.63 ± 0.37[a]
	Dicloran	14	6.60 ± 2.59	0.50 ± 0.33
2. DEN alone		15	8.32 ± 3.71	0.72 ± 0.31
3. Vinclozolin		10	0	0
	Malathion	9	0	0
	DEF	10	0	0
	Chlorobenzilate	8	0	0
	Tecnazene	10	0	0
	Isoproturon	10	0.22 ± 0.27	0.01 ± 0.02
	Dicloran	10	0	0

GST-P positive foci larger than 0.2 mm in diameter were counted.
Significantly different from group 2 at [a]$P < 0.001$, [b]$P < 0.01$ or [c]$P < 0.05$, respectively.

without DEN initiation are summarized in Table II. The number of foci in the control group was $8.32/cm^2$ and the area was 0.72 mm^2/cm^2 Vinclozolin, malathion, chlorobenzilate, tecnazene and isoproturon increased GST-P positive foci development over the control levels in both parameters. Dicloran did not affect foci development, and DEF rather decreased the area of foci.

In the non-DEN-initiated groups, only isoproturon induced a small number of GST-P positive foci larger than 0.2 mm in diameter.

Discussion

Since this assay model is based on the two-step hypothesis of carcinogenesis and on the induction of pre-neoplastic hepatocyte lesions, it primarily provides information on whether a test compound possesses hepato-promoting activity in the rat [6]. However, we think the carcinogenic potential of chemicals can be best assessed by detection of their promoting activity based on the following concepts: (i) most carcinogens possess both tumor-initiating and tumor-promoting activities, and (ii) carcinogenicity and organotropism of carcinogenic agents might be determined mainly by their tumor-promoting activity. Actually more than 90% of the liver carcinogens could be positively detected by this system, although assessment of carcinogens other than established hepatocarcinogens gave less positive results [3–6].

In the present study, five of the seven pesticides were positive: chlorobenzilate, vinclozolin, malathion, tecnazene, and isoproturon. Dicloran was negative in terms of both the number and area of GST-P positive foci, and DEF decreased the area of foci under the present experimental conditions. Dicloran increased the liver weight and was negative in this system. DEF exerted the strongest general toxicity among the seven chemicals; it rather decreased the foci induction at 200 ppm, the lowest dose level among the seven chemicals. Thus the positivities for the pesticides examined were not directly correlated to general and/or liver toxicity of the chemicals as evaluated by the weight data, although some reports indicate that chemicals that increased liver weight enhanced the development of pre-neoplastic foci of the liver [18].

Table III. Comparison of positivity.

Chemical	Chlorinated	Mutagenicity	Carcinogenicity	Liver system
Vinclozolin	+	−	−	
Malathion	−	−	−	
DEF	−	?	?	
Chlorobenzilate	+	−	+	
Tecnazene	+	?	?	
Isoproturon	−	?	?	
Dicloran	+	−	?	

+ Positive or yes; − negative or no; ? unknown.
Mutagenicity in the Ames test. References: [9–16].

The results are compared with their reported mutagenicity in the Ames test and reported carcinogenicity (Table III). Mutagenicity in the Ames test is negative for vinclozolin, malathion, chlorobenzilate, and dicloran, and unknown to us for the other three compounds [8–14,19,20]. Among the seven pesticides, chlorobenzilate has been reported to be carcinogenic, but only in the mouse liver [8–10]. Similar cases of non-mutagenic carcinogens are more commonly observed for the other chlorinated compounds [19,20]. The positive results for malathion and vinclozolin are interesting, as these two chemicals have been evaluated as 'non-carcinogens' in both rats and mice and non-mutagenic in the Ames test [13,19,20]. Carcinogenicity for DEF, dicloran, tecnazene and isoproturon has not fully evaluated as far as we know.

Malathion has been reported as 'non-carcinogenic' in NTP technical reports [11,12]. However, it is worth noting that this agent has demonstrated significant enhancement of the development of several types of tumor in both rats and mice, and the combined incidence of hepatocellular carcinomas and neoplastic nodules in male mice showed a significant linear trend when compared with either the matched controls ($P = 0.041$) or the pooled controls ($P = 0.019$) when administered at concentrations of 8000 and 16 000 ppm in the diet. Although liver carcinogenicity was not evident for malathion in either Osborne Mendel or Fischer rats, our results suggest that malathion possesses tumor-promoting activity in the liver. Vinclozolin has also been reported to be non-carcinogenic by the FAO and WHO [13]; however, an apparent increase in liver weight was noted for both rats and mice to which the chemical was administered at concentrations of up to 4370 ppm in the diet. The level of positivity in this system was almost the same as that of phenobarbital at 0.05% in the diet. Phenobarbital has been considered a typical hepatopromoter and recently evaluated as a liver carcinogen [21].

There seems to be a discrepancy, therefore, between the reported hepatocarcinogenicity in the rat and the positivity in the present system for three chemicals. We consider, however, that these three compounds are possible hepatopromoters in the rat, and this activity has not been fully demonstrated in the long-term carcinogenicity study in this species.

It is possible to conclude that chemicals exerting a positive result in this system might possess hepatopromoting activity even if the hepatocarcinogenicity has not been demonstrated in a long-term study in the rat. In the strategy for cancer prevention, we believe that a battery of rapid tests for carcinogenicity in the liver has an important role to play.

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The genetic and non-genetic toxicity of the fungicide Vinclozolin

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The mutagenic/cocarcinogenic potential of the fungicide Vinclozolin was assessed by a comprehensive examination of toxicity mechanisms at both the genetic and the metabolic level. Vinclozolin did not induce any significant increase in chromosomal aberrations in human pheripheral blood lymphocytes cultured *in vitro*, both in the presence and in the absence of metabolic activation. However, significant dose-related increases in micronucleated erythrocytes (up to 4-fold over the control) were found in the bone marrow cells of mice 24 h after treatment with the fungicide over a range of concentrations from 312.5 to 1250 mg/kg. The morphology and the size of micronuclei induced was suggestive of a predominantly clastogenic mode of action. Several cytochrome P450 (CYP)-dependent reactions have been monitored in liver, kidney and lung microsomes of male and female Swiss Albino CD1 mice in order to ascertain certain toxic non-genetic properties (related to carcinogenesis) of Vinclozolin. It was found to be a selective inducer towards CYP 3A (liver, kidney) and 2E1 (liver), as exemplified by the significant increases of the demethylation of aminopyrine (APND, up to 2.3-fold, female liver), and hydroxylation of *p*-nitrophenol (pNPH, up to 5.6-fold, male liver). In general, however, Vinclozolin has a complex pattern of induction and suppression of CYP-dependent enzymes, as shown from the reduced expression of various monooxygenases depending upon dose, sex or organ considered. For example, pNPH activity was suppressed in kidney (up to 48% loss, averaged between male and female), whereas ethoxycoumarin *O*-deethylase was reduced in lung up to 53% in male (at the highest dose). These data were sustained by means of Western immunoblotting using rabbit polyclonal antibodies anti-CYP 3A and 2E1. Northern blotting analysis using CYP 3A1/2 and 2E1 cDNA biotinylated probes showed that the expression of such isozymes is regulated at the mRNA level. Taken together, the findings indicate the clastogenic activity and the possible cotoxic, cocarcinogenic and promoting potential of Vinclozolin.

Introduction

There are a number of possible ways which humans ca
exposed to pesticides, and thus the to c effects of pestic les
may have consequences for consumer of food, as well a or
production workers, formulators and ther applicators. he
effects of pesticide residues in food and water probably cause the greatest public concern. In particular, there has been concern over the ability of fungicides to induce tumours in the human population (US National Research Council, 1987).

(RS)-3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione (Vinclozolin) is a selective contact fungicide mainly used on stone fruits, strawberries, vegetables and vines.

The pesticide is not a teratogen [Environmental Protection Agency (EPA), 1987]. Reproductive toxicology studies by BASF AG showed the induction of Leydig cell tumours and atrophic ventral prostate and seminal vesicles following chronic exposure of adult male rats to Vinclozolin (van Ravenzwaay, 1992). In a more recent study, the fungicide was demonstrated to possess antiandrogenic activity, that led to developmental toxicity in male rats (Kelce *et al.*, 1994). Vinclozolin has been reported to be non-carcinogenic by the Food and Agriculture Organisation/World Health Organisation (FAO/WHO, 1986). However, questionable results have been obtained in the mouse, in which Vinclozolin causes leukaemia/lymphoma type tumours in male animals and also some adenoma in the lung in female mice (EPA, 1985).

The mutagenicity of Vinclozolin indicates a general tendency to give negative results in the majority of assays. The EPA's files reported that an Ames test, a Chinese hamster sister chromatid exchange (SCE) study, an host-mediated assay with *Salmonella typhimurium*, and a dominant lethal assay in mice were submitted for registration and were negative (EPA, 1985).

From the few data available in the open literature, positive results have been obtained by Chiesara *et al.* (1982) in *S.typhimurium* and in *Schizosaccharomyces pombe*, and Vinclozolin was found to increase the frequency of mitotic recombination and to induce mitotic non-disjunction in diploid colonies of *Aspergillus nidulans* (Georgopoulos *et al.*, 1979; Vallini *et al.*, 1983). No haemoglobin adducts could be found in rats treated with Vinclozolin (Sabbioni and Neumann, 1990).

The above considerations prompted us to improve knowledge on the possible carcinogenic risk to humans of Vinclozolin.

It has been suggested that the outstanding problems faced in the prediction of potential carcinogens are mainly toxicological, non-genetic (Ashby, 1993). For this purpose, recently we developed a multibiomarker approach which combines cytogenetic assays with biochemical and metabolism studies (Cantelli-Forti *et al.*, 1993; Hrelia *et al.*, 1994a). In particular, we proposed a comprehensive examination of the mechanisms of toxicity down to the metabolic level as a possible surrogate measure for promotional and cocarcinogenic activity (Paolini *et al.*, 1994). This approach permits the detection of peculiar effects associated with various stages of cancer development (initiation and promotion). These effects, investigated by modulating specific CYPs, altering the overall metabolic status, can affect the biotransformation of other xenobiotics (i.e. administered drugs, environmental pollutants, etc.), as well as experimental *in vivo* genotoxic response.

Little information is available on the effects of Vinclozolin on CYP isoforms in mammals (Ronis *et al.*, 1994); neither has any characterization of the mammalian induction response been conducted at the molecular level.

Therefore, we determined the capability of Vinclozolin to modulate specific CYP isoforms in liver, kidney and lung of treated mice, and expanded genotoxicity studies, using as end-points micronucleus induction in bone marrow cells of mice, and also chromosome damage in human lymphocytes exposed in culture. The results will permit the appraisal of the chain of events leading to mutagenic and/or carcinogenic effects by Vinclozolin.

Materials and methods

Chemicals

$NADP^+$, G6P, NADPH, 7-ethoxyresorufin, Histopaque-1077 and Acridine Orange were purchased from Sigma Chemical Co (St Louis, MO, USA), G6PDH and phytohaemaglutinin were from Boehringer (Mannheim, Germany), pentoxyresorufin from Molecular Probes (Eugene, OR, USA), rabbit polyclonal antibody anti-CYP 2E1 and 3A1/2 were purchased from Oxygene (Dallas, TX, USA) and Vinclozolin (chemical purity 99.5%) from Lab Service (Bologna, Italy). All other chemicals and solvents were of analytical grade highest purity commercially available.

Chromosome aberration analysis in human lymphocytes in vitro

Blood was obtained from two healthy donors by venous puncture, collected into heparinized tubes and gently shaken. The cultures were set up in duplicate using 2×10^6 lymphocytes, previously isolated by density gradient in 10 ml Roswell Park Memorial Institute (RPMI) tissue culture medium (Gibco, UK), containing 20% fetal bovine serum (Gibco), 1% phytohaemagluunin, glutamine 1 mM (Gibco) and 500 IU of penicillin and streptomycin (Gibco), and incubated at 37°C and 5% CO_2 for 72 h. After 48 h of incubation, the cultures were treated with Vinclozolin, dissolved in dimethyl sulphoxide (DMSO), in the presence and in absence of phenobarbital plus β-naphthoflavone-induced rat liver S9 microsomal fraction (Cantelli-Forti *et al.*, 1984). A fortified mixture containing 4 mM $NADP^+$, 5 mM G6P, 8 mM $MgCl_2$ and 33 mM KCl in 100 mM phosphate buffer (pH 7.4) was added to S9 prior to experiments (Cantelli-Forti *et al.*, 1984). The dose levels for Vinclozolin, tested up to the solubility limit, were 1, 3, 10, 30, 100 μg/ml culture. The positive control substances were ethylmethanesulphonate (120 μg/ml) and cyclophosphamide (60 μg/ml) in the experiments in the absence and in the presence of metabolic activation, respectively. The lymphocyte cultures without S9 mix were treated with Vinclozolin for 24 h, while the lymphocyte cultures with S9 mix were exposed to the test compound only for 3 h, after which the growth medium was replaced with fresh medium. Cultures were treated with colcemide (final concentration 0.1 μg/ml) 2 h prior to harvest (harvest time: 24 h after treatment). Hypotonic treatment for 10 min in 0.075 M KCl was followed by fixation with methanol/glacial acetic acid (3:1). The slides were stained in 5% Giemsa for 5 min.

Chromosome aberrations (CAs) were scored in 100 metaphases each donor, 200 metaphases per sample, according to the classification criteria suggested by Savage (1976). Gap aberrations were excluded from the total number of CAs and considered separately. The number of aberrant metaphases, referred to in the text as damaged cells, and the number of CAs were counted.

Animals

Male and female Swiss Albino CD1 mice (Nossan, Correzzana, Milan, Italy), weighing 28–30 g, were used in the experiments. They were housed under controlled conditions (12 h light/dark cycle, 22°C, 60% humidity), fed a rodent chow (Nossan) and had tap water *ad libitum*.

Micronuclei (MN) analysis

Five male mice for each treatment were treated i.p. with different single doses of Vinclozolin in corn oil, corresponding to 50, 25 and 12.5% of LD_{50}. LD_{50} was previously determined using six animals for each dose, eight doses, and calculated using the Litchfield–Wilcoxon method (data not shown). Animals treated with corn oil represented the solvent control; cyclophosphamide (60 mg/kg b.w.) and vincristine (0.1 mg/kg b.w.) were the reference clastogen and the reference aneuploidogen respectively. Mice were killed humanely 24 h after treatment, in accordance with approved Home Office procedures appropriate to the species. The frequency of MN was evaluated in polychromatic erythrocytes obtained by column fractionation of bone marrow cells, according to Vigagni and Norppa (1995). Briefly, femoral cells were flushed out with fetal calf serum (Gibco); the cellular suspension was added on a 2.0 cm height cellulose column (type 50) and washed out with Hank's Balanced Salt Solution (HBSS, Gibco) with a 15 drop/min speed. The collected cells were sedimented by centrifugation (1800 r.p.m., 5 min) and resuspended in fetal calf serum. The slides were prepared by cytocentrifugation, air-dried and fixed in methanol for 15 min. Slides were stained in a solution of Acridine Orange (12.5 mg/100 ml buffer) for 60 s according to the method of Hayashi *et al.* (1983). Slides were then allowed to stand in buffer for 10 min, the smears were 'wet' mounted using the phosphate buffer and observed for the presence of MN in polychromatic erythrocytes (PCEs). A total of 2000 PCEs were scored per animal and data were summarized as the mean number of micronucleated PCE/1000 PCE. In addition, the ratio between PCE and PCE plus normochromatic erythrocytes (NCE) was also determined. During the scoring, the diameters of the cell (D) and micronucleus (d) in each MNPCE were compared directly under the microscope. If $d < D/4$, the MN was classified as 'normal', while it was classified as 'large' if $d > D/4$. When determination by direct comparison was difficult, the MNPCE was photographed and measured. Furthermore, the MN were classified according to the morphological criteria used by Yamamoto and Kikuchi (1980), i.e. normal, double, multi, ring, crescent and large.

Animal treatment and preparation of subcellular fractions

Prior to experiments, male and female mice were divided randomly in groups of six animals each one. Vinclozolin was suspended in corn oil and administered (i.p.) in a single (625 and 1250 mg/kg) or repeated (750 mg/kg, for 3 consecutive days) dose. Controls received vehicle only, under the same conditions. Mice were starved 16 h prior to death. Liver, kidney and lung were rapidly removed and S9 fraction (9000 *g*) was prepared as previously described (Cantelli-Forti *et al.*, 1984). The post-mitochondrial supernatant was then centrifuged for 60 min at 105 000 *g*, pellet resuspended and centrifuged again for 60 min at 105 000 *g* to give the final microsomal fraction. The resultant washed microsomes were then resuspended with an hand-driven Potter Elvejhem homogenizer in a Tris-HCl buffer containing EDTA and glycerol. Subcellular fractions were immediately frozen in liquid nitrogen (–196°C), stored at –80°C in a deep freezer and used within a week.

CYP content

The determination of CYP was based on its reduced CO-binding spectrum, by the difference between the reduced form plus carbon monooxide and reduced form at 450–490 nm (Omura *et al.*, 1964).

CYP c-reductase activity

The analytical method is based on the determination of the reduction rate of cytochrome c at 550 nm, essentially according to Bruce (1967), with the following modifications: carbon monoxide was used as an inhibitor of cytochrome c-oxidase (cytochrome aa3) present as a contaminant in the S9 fraction, reaction was started when cytochrome c was added in the assay.

Aminopyrine N-demethylase (APND) activity

The activity was determined by quantification of CH_2O release, according to Mazel (1971). The total incubation volume was 3 ml, composed of 0.5 ml of a water solution of 50 mM aminopyrine and 25 mM $MgCl_2$, 1.48 ml of a 0.60 mM $NADP^+$, 3.33 mM G6P in 50 mM Tris–HCl buffer (pH 7.4), 0.02 ml G6PDH (grade II) and 0.125 ml of sample. After 5 min of incubation at 37°C, the yellow colour, developed by the reaction of the released CH_2O with the Nash reagent was read at 412 nm, and the molar absorptivity of 8000 used for calculation (Nash, 1953).

p-Nitrophenol hydroxylase (pNPH) activity

The activity was assayed adding the following reagents in a final volume of 2 ml: 0.10 ml *p*-nitrophenol in 50 mM Tris–HCl buffer (pH 7.4), 5 mM $MgCl_2$ and a NADPH-generating system consisting of 0.4 mM $NADP^+$, 30 mM isocitrate, 0.2 IU of isocitrate dehydrogenase and 0.5 mg of protein (sample). After 10 min at 37°C, the reaction was terminated by the addition of 0.5 ml of a 0.6 N perchloric acid. The precipitated proteins were removed by centrifugation and 1 ml of the resultant supernatant was mixed with 1 ml 10 N NaOH. Immediately after mixing, the absorbance at 546 nm was measured and the 4-nitrocatechol concentration determined utilizing the molar extinction coefficient of 10.28 nM^{-1} cm^{-1} (Reinke *et al.*, 1985).

Pentoxyresorufin O-dealkylase (PROD) and ethoxyresorufin O-deethylase (EROD) activities

Reaction mixture consisted of 2.0 ml 0.05 M Tris–HCl buffer (pH 7.4), 0.025 μM $MgCl_2$, 200 μM pentoxyresorufin, 0.08 ml of sample and 130 mM NADPH. The rate of resorufin formation at 37°C was calculated by comparing the rate of increase in relative fluorescence with the fluorescence of known amounts of resorufin (excitation 522 nm, emission 586 nm) (Reinke *et al.*, 1985). The EROD activity was measured exactly in the same manner as described for PROD activity, except that the substrate (ethoxyresorufin) concentration was 1.7 μM (Lubet *et al.*, 1985).

Ethoxycoumarin O-deethylase (ECOD) activity

The activity was determined by quantification of umbelliferone formation, according to Aitio (1978). The incubation mixture consisted of 2.6 ml

bone marrow cells was not affected in each group and their values were comparable with the control, indicating that the cytogenetic damage results were not compromised by a decrease in bone marrow cell proliferation.

Vinclozolin induced a significant ($P < 0.01$) dose-related increase in the frequency of micronucleated PCEs, with a 4-fold increase in frequency over a range of concentration from 312.5 to 1250 mg/kg. The micronucleus test can detect both chromosomal fragments and lagging chromosomes (Heddle *et al.*, 1991). In attempt to determine the relative contribution of fragments and whole chromosomes to the MN induced by Vinclozolin, the slides for the highest dose of fungicide were re-assessed for MNPCE, and the morphology and size of MN was compared with those induced by the aneugen vincristine and by cyclophosphamide, as a reference clastogen (Figure 1). Morphological data showed that Vinclozolin, like cyclophosphamide, induced aberrant MN of the double, ring and multiple category, in addition to 'normal' MN. Crescent-shaped and large MN were only produced by the reference aneuploidogen vincristine. Results obtained suggest that the majority of MN induced by Vinclozolin were acentric chromosome fragments.

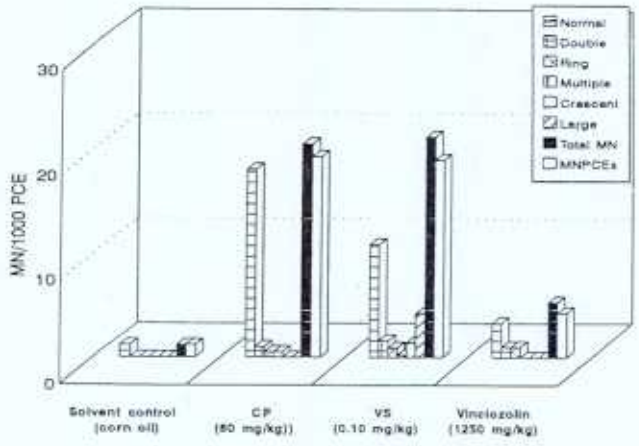

Fig. 1. Comparison of results obtained in the bone marrow of mice treated with Vinclozolin, cyclophosphamide (CP) or vincristine (VS), based on morphological classification of micronuclei (MN) in polychromatic erythrocytes (PCE). Each treatment group contains five mice and 2000 PCE were examined per animal.

Metabolizing enzymes

The expression of phase I-dependent reactions in microsomes of mice receiving 625 or 1250 mg/kg dose of Vinclozolin, is shown in Tables III–V. Various substrates were used as probes of different CYPs, such as pentoxyresorufin (CYP 2B1), ethoxyresorufin (1A1), aminopyrine (3A), *p*-nitrophenol (2E1) and ethoxycoumarin (mixed, mainly 2B1 and 1A1). CYP level and its NADPH-cytochrome P450 (c) reductase activities were also studied.

In general, a single dose of the fungicide did not affect markedly the considered monooxygenases in liver microsomes; CYP content and the reductase were slightly affected in both a positive or negative manner (Table III). Such differences are therefore not relevant from a toxicological point of view.

Table IV shows the behaviour of the oxidase activities in kidneys, where CYP 3A isoforms were significantly ($P < 0.01$) increased, as exemplified by the induction of the APND activity up to 1.76-fold in male animals. On the contrary, a decrease of pNPH activity was observed at both 625 (48% loss, averaged between male and female) and 1250 mg/kg dose (31% loss, averaged between male and female).

Vinclozolin was able to appreciably affect microsomal monooxygenases in lung preparations (Table V), both positively and negatively. For example, a decrease in the deethylation of ethoxycoumarin (up to 53% loss, male, highest dose) was recorded, whereas it was induced in female mice (2-fold, highest dose). A decreased pNPH activity was also seen in female (up to 13%, lowest dose), whereas an induction effect in male mice was recorded.

However, the observed CYP changes after a single treatment of fungicide were generally moderate. Therefore, the effects of Vinclozolin at 750 mg/kg daily for 3 consecutive days on CYP-dependent enzymes were studied (Tables VI and VII). This protocol was chosen in order to magnify the inductive phenomenon, since it was not possible to increase the level of the administered dose.

Table VI shows the expression of metabolizing enzymes in liver of treated mice. The induction of ECOD (up to 4.58-

Table III. Expression of carcinogen metabolizing enzymes in hepatic microsomes from Vinclozolin-treated mice

Parameters	Untreated (corn oil only)		Vinclozolin (625 mg/kg b.w.)		Vinclozolin (1250 mg/kg b.w.)	
	Male	Female	Male	Female	Male	Female
Pentoxyresorufin *O*-Dealkylase (2B1) (pmol×mg^{-1} × min^{-1})	39.68 ± 3.85	22.42 ± 2.38	29.15 ± 2.76*	39.77 ± 5.17*	23.84 ± 3.30*	40.02 ± 7.53*
Ethoxyresorufin *O*-deethylase (1A1) (pmol×mg^{-1} × min^{-1})	54.24 ± 4.89	53.91 ± 5.12	61.09 ± 5.96**	52.91 ± 5.56	37.80 ± 3.86*	50.27 ± 4.38
Ethoxycoumarin *O*-deethylase (mixed) (nmol×mg^{-1} × min^{-1})	0.72 ± 0.11	0.86 ± 0.12	0.84 ± 0.20	1.18 ± 0.21**	0.62 ± 0.18	1.41 ± 0.13*
Aminopyrine *N*-demethylase (3A) (nmol×mg^{-1} × min^{-1})	8.12 ± 0.94	9.34 ± 1.77	11.60 ± 1.23*	11.06 ± 1.32	10.88 ± 1.34**	13.31 ± 1.52*
p-nitrophenol hydroxylase (2E1) (nmol×mg^{-1} × min^{-1})	1.57 ± 0.12	1.51 ± 0.13	1.67 ± 0.15	1.53 ± 0.14	1.06 ± 0.09*	1.21 ± 0.11*
Cytochrome P450 (nmol×mg^{-1})	0.53 ± 0.06	0.54 ± 0.06	0.78 ± 0.08*	0.50 ± 0.04	0.99 ± 0.12*	0.30 ± 0.02*
CYP c-reductase (nmol×mg^{-1} × min^{-1})	48.00 ± 6.11	57.59 ± 6.64	55.08 ± 7.42	67.18 ± 9.75**	62.19 ± 8.39**	62.68 ± 8.80

Each value represents the mean ± SD of six independent experiments, each done on single animal.
See Materials and methods section for details and experimental procedures.
**$P < 0.05$, *$P < 0.01$, with respect to controls, using Wilcoxon's rank method.

Table I. Induction of chromosome aberrations in cultured human lymphocytes treated with Vinclozolin[a]

Treatment μg/ml	S9 mix	Donor A					Donor B					Total mean (X ± SE)	
		G	B'	B"	F	RR	G	B'	B"	F	RR	%CA	%DC
Negative control	–	4	1	0	0	0	2	0	0	1	0	1.0 ± 0.0	1.0 ± 0.0
Solvent control (DMSO)	–	1	0	0	2	0	3	1	0	0	0	1.5 ± 0.5	1.0 ± 0.0
Positive control (EMS 120)	–	9	6	2	11	1	8	8	1	7	1	18.5 ± 1.5[b]	18.5 ± 1.5[b]
Vinclozolin													
	–	0	0	0		0	1	1	0	0	0	1.0 ± 0.0	1.0 ± 0.0
3	–	3	0	0	1	0	0	0	0	0	0	0.5 ± 0.5	0.5 ± 0.5
10	–	5	1	0	0	0	4	0	0	0	0	0.5 ± 0.5	0.5 ± 0.5
30	–		1	0	0	0	2	0	0	4	0	2.5 ± 1.5	2.5 ± 1.5
100	–	4	1	0	0	0	3	0	0	0	0	0.5 ± 0.5	0.5 ± 0.5
Negative control	+	2	0	0	0	0	4		0	0	0	0.5 ± 0.5	0.5 ± 0.5
Solvent control (DMSO)	+			0	0	0		0	0	2	0	1.5 ± 0.5	1.5 ± 0.5
Positive control (CP 60)	+	8		3	3		9	13		6	0	17.0 ± 3.0[b]	17.0 ± 3.0[b]
Vinclozolin													
	+	3	0	0		0	4	0	0	1	0	1.0 ± 0.0	1.0 ± 0.0
3	+	1	2	0		0	2	0	0	0	0	1.5 ± 1.5	1.5 ± 1.5
10	+	6		0	0	0	2	0	0	0	0	0.5 ± 0.5	0.5 ± 0.5
30	+	2	0	0		0	7	3	0	0	0	2.0 ± 1.0	2.0 ± 1.0
100	+	4		0	0	0	3	0	0	1	0	1.0 ± 0.0	1.0 ± 0.0

DMSO = dimethyl sulphoxide; EMS = ethylmethane sulphonate; CP = cyclophosphamide.
[a]A total of 200 metaphases per treatment (100 metaphases each donor) was scored. G, gaps; B', chromatid breaks; B", isochromatic breaks; F, fragments; RR, chromosomal rearrangements; %CA, percentage of chromosome aberrations without gaps; %DC, percentage of cells with at least one chromosome aberration but not gaps.
[b]$P < 0.01$ difference against solvent control according to the χ^2 test.

composed of 1 mM ethoxycoumarin, 5 mM $MgCl_2$, 2 ml of an NADPH-generating system (see APND) and 25 μMl of sample. After 5 min of incubation at 37°C, the reaction was stopped by adding 85 μM of 0.31 M trichloroacetic acid. The pH of the mixture was brought to ~10 by adding 0.65 ml of NaOH–glycine buffer, pH 10.3; the amount of umbelliferone was measured fluorimetrically (excitation 390 nm; emission 440 nm).

Protein concentration

Protein concentration was determined according to the method of Lowry (1951) and Bayley (1967), using bovine serum albumin as a standard and diluting samples 1000 times to provide a suitable protein concentration.

Electrophoresis and Western immunoblot

Microsomes from both control and Vinclozolin treated mice were solubilized in sodium dodecyl sulphate (SDS) and resolved by polyacrylamide gel electrophoresis (PAGE) (Laemmly, 1970; Towbin *et al.*, 1979). Western blot analysis with polyclonal antibody (anti-CYP 2B1 and 3A) raised against rat hepatic CYPs 2B1 and 3A (Dutton *et al.*, 1989) was performed using hepatic microsomes (0.025 mg of microsomal protein were electrophoresed each time) and visualized with 4-chloro-1-naphthol in a 0.006% hydrogen peroxide solution.

RNA isolation and Northern hybridization

RNA was isolated from mouse liver tissue according to Chomczynski and Sacchi (1987), and dissolved in 50 μM SDS 0.5% at 65°C for 10 min to be used for Northern blotting.

tRNA was separated according to size by electrophoresis and transferred to a nylon membrane, prehybridized in 6 ml of hybridization buffer for at least 4 h; cDNA 2E1 and 3A probes were labelled by using Bioprime DNA labelling system. Hybridization with biotinylated probes was performed at 68°C overnight, and reaction with streptavidin–alkaline phosphatase conjugate was used for identification (Sambrook *et al.*, 1989).

Statistics and computer analysis

Genotoxicity results were analysed by using the analysis of variance (one-way) and the χ^2 test. Statistical analysis on biochemical data was performed using Wilcoxon's rank method as reported by Box and Hunter (1978).

Results

Chromosomal analysis

The data of chromosomal analysis of human lymphocytes, obtained from two different donors, exposed *in vitro* to Vinclozolin at five subtoxic concentrations, are shown in Table I.

Treatment of human lymphocytes with different doses of Vinclozolin, ranging from 1 to 100 μg/ml, both in the presence and in the absence of microsomal S9 fraction obtained from induced rat liver, did not increase the number of CAs significantly per cell or the percentage of aberrant metaphases in any concentration tested. The aberrations encountered were mainly chromatid break-type aberrations. The range of CAs of treatment groups (0.5–2.5%) were comparable with control groups (0.5–1.5%), both in the absence and in the presence of S9 mix. The mitotic indices of all the cultures were approximately the same, with no relation to dose (data not shown).

Table II. Frequency of polychromatic erythrocytes (PCE) with micronuclei (MN) in mice treated with Vinclozolin

Treatment (mg/kg b.w.)	PCE with MN/1000 cells[a]	PCE/PCE + NCE[b]
Negative control	0.8 ± 0.6	65.2 ± 4.9
Solvent control (corn oil)	1.2 ± 0.6	69.6 ± 1.5
Positive control (CP 60)	20.4 ± 4.3[c]	66.4 ± 2.6
Vinclozolin		
312.5	1.2 ± 0.2	70.0 ± 0.6
625.0	3.4 ± 0.5[d]	78.0 ± 5.2
1250	5.3 ± 1.0[c]	67.0 ± 4.6

[a]Each treatment group contains five mice and 2000 PCE were examined per animal. Data indicate mean ± SE.
[b]Data indicate the percentage mean ± SE of PCE valued on 1000 erythrocytes. NCE = hormochromatic erythrocytes.
[c]Significantly different from the solvent control group ($P < 0.01$, χ^2 test).
[d]Significantly different from the solvent control group ($P < 0.05$, χ^2 test).

Micronucleus analysis

Data from a preliminary time-course study on the genotoxic effects of Vinclozolin in bone marrow cells of mice showed that Vinclozolin induced the major MN frequency at 24 h after treatment (data not shown). For this reason 24 h was the chosen sampling time.

Data of MN analysis after treatment of mice with three different doses of Vinclozolin from 312.5 to 1250 mg/kg b.w. are shown in Table II.

There was no significant toxicity in any of the animals exposed to Vinclozolin. The PCE/PCE + NCE ratio of the

Table VII. Expression of carcinogen metabolizing enzymes in kidney microsomes from Vinclozolin-treated mice (3 days)

Parameters	Untreated (corn oil only) for 3 days		Vinclozolin (750 mg/kg b.w.) for 3 days	
	Male	Female	Male	Female
Pentoxyresorufin *O*-dealkylase (2B1) (pmol×mg^{-1} × min^{-1})	10.35 ± 1.12	15.64 ± 1.65	18.27 ± 1.76*	19.07 ± 2.12**
Ethoxyresorufin *O*-deethylase (1A1) (pmol×mg^{-1} × min^{-1})	13.86 ± 1.42	15.96 ± 1.53	12.12 ± 1.31	16.84 ± 1.54
Ethoxycoumarin *O*-deethylase (mixed) (nmol×mg^{-1} × min^{-1})	0.89 ± 0.10	0.71 ± 0.08	0.79 ± 0.08	0.78 ± 0.07
Aminopyrine *N*-demethylase (3A) (nmol×mg^{-1} × min^{-1})	1.99 ± 0.23	1.05 ± 0.09	2.76 ± 0.25*	2.02 ± 0.23*
p-nitrophenol hydroxylase (2E1) (nmol×mg^{-1} × min^{-1})	0.59 ± 0.07	0.42 ± 0.03	0.24 ± 0.03*	0.18 ± 0.02*

Each value represents the mean ± SD of six independent experiments, each done on single animal. See Materials and methods section for details and experimental procedures.
**$P < 0.05$; *$P < 0.01$, with respect to controls, using Wilcoxon's rank method.

between male and female) (Table VII). Lung CYP-dependent enzymes were only slightly affected by Vinclozolin treatment (data not shown).

CYP induction was corroborated by means of Western immunoblotting analysis using rabbit polyclonal antibodies anti CYP 3A1/2 (liver), and 2E1 (liver). Representative Western blots, with typical CYP-induced signals (51 and 52 kDa bands for 3A and 2E1 respectively), were reported in Figures 2 and 3. The two inducible apoprotein bands recognized in liver with antibody anti-CYP 3A1/2 (Vinclozolin, lane 3 compared with PCN, lane 1) and anti-CYP 2E1 (lane 3 compared with ETOH, lane 1), were shown in Figures 2 and 3 respectively.

The increased signals obtained by Northern blot analysis show that the amount of hybridizable mRNA is increased after Vinclozolin treatment in comparison with controls for both CYPs 3A and 2E1 (Figures 4 and 5 respectively). This seems to suggests that the mechanism of CYP 3A and 2E1 induction by Vinclozolin affects the rate of protein synthesis regulating the expression at the mRNA level. Whereas 3A induction seems to reflect the classical glucocorticoid receptor pathway, the 2E1 one is like to that expressed by high doses of ethanol.

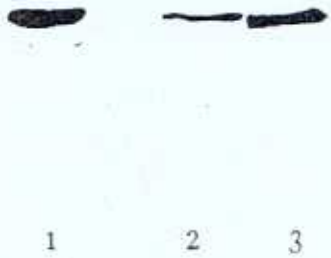

Fig. 2. Western blot analyses of female liver microsomes from control (lane 2, corn oil only), Vinclozolin (lane 3, 750 mg/kg b.w., daily for 3 consecutive days) or pregnenolone 16-α carbonitrile (PCN, 100 mg/kg b.w.)-treated CD1 mice. Microsomal proteins (22 μg) were applied to the wells and probed with anti-CYP 3A1/2 as described in the Materials and methods section.

Fig. 3. Western blot analysis of male liver microsomes from control (lane 2, corn oil only), Vinclozolin (lane 3, 750 mg/kg b.w. daily, for 3 consecutive days) or ethanol-treated (lane 1, 15% v/v, 21 days) CD1 mice. Microsomal proteins (22 μg) were applied to the wells and probed with anti-CYP 2E1 as described in the Materials and methods section.

Discussion

On the basis of previous reports in the literature, Vinclozolin seems to be relatively 'non-toxic' (EPA, 1985, 1987). The fungicide is not a teratogen, and mutagenicity testing failed to show genotoxicity in bacterial, cell culture and whole animal assays. However, there have been reports of a weak oncogenic effect in mice and reproductive and developmental toxicity in rats (EPA, 1985; van Ravenzwaay, 1992; Kelce *et al.*, 1994).

Our *in vitro* study did not reveal any significant increase in chromosome aberrations in lymphocyte cultures treated with Vinclozolin. These results are in agreement with early data on mutagenicity, and may suggest that Vinclozolin is a non-genotoxic compound. However, in contrast to the negative effects previously seen, Vinclozolin has been proved to be an inducing agent in the mouse bone marrow micronucleus assay. The *in vivo* study demonstrated a dose–response relationship between exposure to Vinclozolin and MN induction in bone marrow cells of treated mice, with statistically elevated frequency over control at concentration of 625 and 1250 mg/kg b.w.

It was recently found that certain detrimentral effects of Vinclozolin administration in rats, as developmental toxicity, is dependent on metabolic conversion into metabolites which possess anti-androgenic activity (Kelce *et al.*, 1994). The positive clastogenic response *in vivo* of Vinclozolin in the present study, should be derived by either metabolites produced directly in the bone marrow or in other tissues, since the fungicide is able to modulate its own metabolism. The failure of Vinclozolin to induce clastogenic damage *in vitro* but not *in vivo* may reflect a deficiency of the S9 mix in producing potentially reactive species important to the genotoxic response.

On the other hand, the activity of Vinclozolin as a bone marrow MN-inducing agent may indicate specific toxicity to metaphase spindles as opposed to chromosomes. This hypothesis is supported by earlier observations on mitotic instability and mitotic non-disjunction effects in lower eukaryotes by Vinclozolin (Georgopoulos *et al.*, 1979; Vallini *et al.*, 1983).

fold, male) was accompained by the increased of the CYP 3A (up to 2.29-fold, female) and the 2E1 (up to 5.6-fold, male) dependent reactions. CYP content and reductase were also significantly increased ($P < 0.01$), mainly in female.

A selective induction of 3A isoforms, probed by the demethylation of aminopyrine, was observed in kidney, whereas hydroxylation of *p*-nitrophenol was significantly ($P < 0.01$) reduced in both sexes (up to 42% loss, averaged

Table IV. Expression of carcinogen metabolizing enzymes in kidney microsomes from Vinclozolin-treated mice

Parameters	Untreated (corn oil only)		Vinclozolin (625 mg/kg b.w.)		Vinclozolin (1250 mg/kg b.w.)	
	Male	Female	Male	Female	Male	Female
Pentoxyresorufin *O*-dealkylase (2B1) (pmol×mg^{-1} × min^{-1})	23.46 ± 3.14	20.36 ± 2.56	27.80 ± 4.19	24.06 ± 2.38	30.22 ± 3.15*	25.15 ± 2.60**
Ethoxyresorufin *O*-deethylase (1A1) (pmol×mg^{-1} × min^{-1})	14.91 ± 1.45	16.20 ± 1.58	13.93 ± 1.42	18.50 ± 1.79	13.36 ± 1.28	16.78 ± 1.61
Ethoxycoumarin *O*-deethylase (mixed) (nmol×mg^{-1} × min^{-1})	0.37 ± 0.04	0.63 ± 0.05	0.33 ± 0.02	0.62 ± 0.09	0.35 ± 0.05	0.71 ± 0.06
Aminopyrine *N*-demethylase (3A) (nmol×mg^{-1} × min^{-1})	2.71 ± 0.32	2.09 ± 0.22	4.78 ± 0.51*	2.56 ± 0.28	4.76 ± 0.52*	2.16 ± 0.22
p-nitrophenol hydroxylase (2E1) (nmol×mg^{-1} × min^{-1})	0.36 ± 0.03	0.38 ± 0.03	0.14 ± 0.01*	0.25 ± 0.02*	0.20 ± 0.02*	0.32 ± 0.02*

Each value represents the mean ± SD of six independent experiments, each done on single animal.
See Materials and methods section for details and experimental procedures.
**$P < 0.05$; *$P < 0.01$, with respect to controls, using Wilcoxon's rank method.

Table V. Expression of carcinogen metabolizing enzymes in lung microsomes from Vinclozolin-treated mice

Parameters	Untreated (corn oil only)		Vinclozolin (625 mg/kg b.w.)		Vinclozolin (1250 mg.kg b.w.)	
	Male	Female	Male	Female	Male	Female
Pentoxyresorufin *O*-dealkylase (2B1) (pmol×mg^{-1} × min^{-1})	26.60 ± 4.15	25.85 ± 3.12	31.18 ± 5.93	23.15 ± 2.51	30.68 ± 4.16	23.61 ± 2.23
Ethoxyresorufin *O*-deethylase (1A1) (pmol×mg^{-1} × min^{-1})	34.81 ± 2.89	20.92 ± 2.58	49.20 ± 5.32*	20.84 ± 2.12	27.89 ± 2.65*	26.33 ± 3.21**
Ethoxycoumarin *O*-deethylase (mixed) (nmol×mg^{-1} × min^{-1})	0.32 ± 0.09	0.13 ± 0.01	0.18 ± 0.02*	0.20 ± 0.01*	0.15 ± 0.02*	0.26 ± 0.03*
Aminopyrine *N*-demethylase (3A) (nmol×mg^{-1} × min^{-1})	1.35 ± 0.12	1.44 ± 0.17	1.39 ± 0.11	1.51 ± 0.18	1.44 ± 0.19	1.42 ± 0.13
p-nitrophenol hydroxylase (2E1) (nmol×mg^{-1} × min^{-1})	0.76 ± 0.07	0.68 ± 0.05	0.84 ± 0.07*	0.59 ± 0.04*	0.93 ± 0.08*	0.65 ± 0.07 *

Each value represents the mean ± SD of six independent experiments, each done on single animal.
See Materials and methods section for details and experimental procedures.
**$P < 0.05$; *$P < 0.01$, with respect to controls, using Wilcoxon's rank method.

Table VI. Expression of carcinogen metabolizing enzymes in hepatic microsomes from Vinclozolin-treated mice (3 days)

Parameters	Untreated (corn oil only) for 3 days		Vinclozolin (750 mg/kg b.w.) for 3 days	
	Male	Female	Male	Female
Pentoxyresorufin *O*-dealkylase (2B1) (pmol×mg^{-1} × min^{-1})	39.06 ± 4.51	29.92 ± 2.69	13.94 ± 1.56*	26.92 ± 2.56**
Ethoxyresorufin *O*-deethylase (1A1) (pmol×mg^{-1} × min^{-1})	35.98 ± 3.46	26.12 ± 2.45	51.77 ± 5.32*	23.67 ± 2.64**
Ethoxycoumarin *O*-deethylase (mixed) (nmol×mg^{-1} × min^{-1})	0.62 ± 0.05	1.00 ± 0.13	2.84 ± 0.31*	1.84 ± 0.22*
Aminopyrine *N*-demethylase (3A) (nmol×mg^{-1} × min^{-1})	7.89 ± 0.84	13.00 ± 1.27	15.33 ± 1.69*	29.77 ± 2.77*
p-nitrophenol hydroxylase (2E1) (nmol×mg^{-1} × min^{-1})	1.02 ± 0.26	1.44 ± 0.13	5.71 ± 0.25**	3.40 ± 0.15*
Cytochrome P450 (nmol×mg^{-1})	0.54 ± 0.04	0.45 ± 0.04	1.15 ± 0.16*	1.86 ± 0.21*
Cyp c-reductase (nmol×mg^{-1} × min^{-1})	40.13 ± 3.95	42.85 ± 4.06	35.02 ± 3.48**	69.57 ± 7.34**

Each value represents the mean ± SD of six independent experiments, each done on single animal.
See Materials and methods section for details and experimental procedures.
**$P < 0.05$; *$P < 0.01$, with respect to controls, using Wilcoxon's rank method.

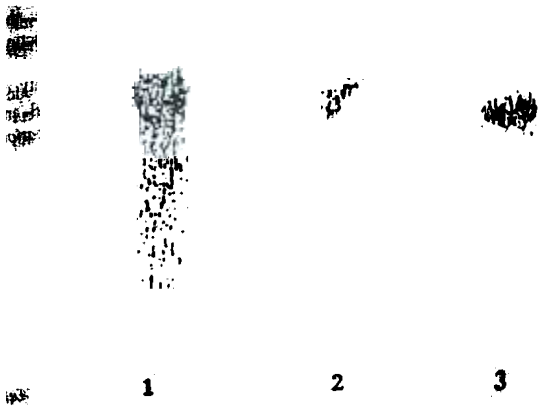

Fig. 4. Northern blot analysis of mRNA from liver of control (lane 2, corn oil only), pregnenolone 16-α carbonitrile (lane 1, 100 mg/kg b.w.) or Vinclozolin-treated (lane 3, 750 mg/kg b.w. daily, for 3 consecutive days) CD1 mice. Total mRNA (20 μg) from each sample were electrophoresed and probed with a full-length cDNA 3A.

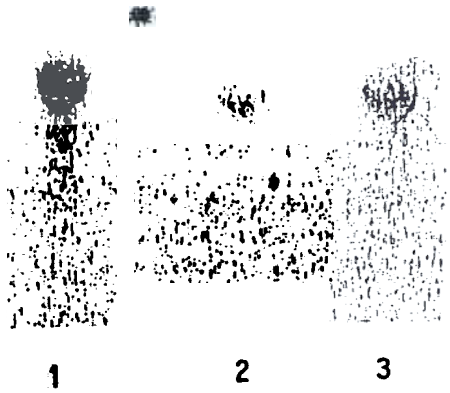

Fig. 5. Northern blot analysis of mRNA from liver of control (lane 2, corn oil only), ethanol (lane 1, 15% v/v, for 3 weeks, *ad libitum*) or Vinclozolin-treated (750 mg/kg b.w. daily, for 3 consecutive days) CD1 mice. Total mRNA (20 μg) from each sample were electrophoresed and probed with a full-length cDNA 2E1.

The role of aneuploidy as a marker of carcinogenicity is still questionable. Changes in chromosome number are rarely associated with initiating events, but generally with the karyotipic instability in the later stages of tumorigenesis (de G.Mitchell *et al.*, 1995). However, induction of aneugenic events could present a significant genetic disturbance in the germ and somatic cells of mammals.

In our study, the morphology and size of MN induced by Vinclozolin is suggestive of a predominantly clastogenic mode of action. Crescent shaped and large MN are in general specific to aneugenic compounds (Tinwell and Ashby, 1991), and the slides for Vinclozolin showed clear induction of other classes of aberrant MN (double, multiple, rings), indistinguishable from those present in the cyclophosphamide-treated animal group, which represents the clastogen control. However, since the fraction technique used in the present study and the morphological characterization of the MN may understimate the effects of aneuploidogens (Vigagni and Norppa, 1995), it is still possible that Vinclozolin is an aneugen but not deteced with the reported assay.

Activity observed for Vinclozolin in the bone marrow micronucleus assay give a strong indication that the fungicide is an *in vivo* genotoxin. An interesting implication of this is that Vinclozolin may initiate carcinogenesis in tissues which may be promoted into sites of carcinogenesis (Ashby, 1992).

Vinclozolin has been shown to exhibit other toxicological effects, which may be relevant to the process of carcinogenesis *in vivo*.

A cell transforming activity was reported by Perocco *et al.* (1993) in the BALB/c 3T3 assay. Since the cell transformation assay can detect both carcinogens that act by a primary genotoxic mechanism and those that act by alternative non-genotoxic mechanisms [Lubet *et al.*, 1990; International Agency for Research on Cancer (IARC), 1991], transforming activity may indicate that Vinclozolin may promote to a more malignant phenotype a background of cells initiated by the fungicide itself, or present in the BALB/c 3T3 population. A weak hepatopromoting or hepatocarcinogenic activity of Vinclozolin, even if hepatocarcinogenicity has not been established, has been suggested by Ito *et al.* (1994) who found a significant increase in the rate of preneoplastic glutathione S-transferase placental form positive foci in the rat liver.

In the present study, Vinclozolin exhibited a complex pattern of induction and suppression of CYP isozymes, typical of ergosterol biosynthesis inhibiting fungicides (Ronis *et al.*, 1994). In particular, it was found to selectively induce murine CYP 2E1 isoforms in liver microsomes, as exemplified by the significant increase in the hydroxylation of *p*-nitrophenol, and corroborated by Western blotting, which is related to the induction specificity. The amount of 2E1 cDNA recognizable mRNA was increased in Vinclozolin treated mice, probably due to stimulated transcription or stabilization of mRNA (Kraner *et al.*, 1993). The xenobiotic-mediated induction process under conditions where the 2E1 enzymes were induced (for acetone or ethanol treatment) is different (involving protein stabilization) from the acute phase of 2E1 induction (Song *et al.*, 1989). However, at high doses, also the typical 2E1 inducers are able to increase the enzymatic activity by mRNA modulation.

Vinclozolin was also a selective inducer of CYP 3A in both liver and kidney subcellular preparations, as recorded by the marked increase of the APND activity and sustained by immunoblotting analysis. Again, Northern blotting showed that the amount of 3A cDNA recognizable mRNA was increased in Vinclozolin treated CD1 mice, suggesting that the expression of such isoform is regulated at mRNA level. The observed sex dependent differences in enzyme activation/suppression could be explained in terms of quantitative or qualitative differences in the isozymes, which are under the control of sex hormones.

In general, Vinclozolin, is able to modulate the activity of several CYPs and should be considered an 'unspecific' enzyme inducer or suppressor. However, its activity is preferential toward 2E1 and 3A CYPs. To our knowledge this is the first evidence of such specific induction by Vinclozolin in liver and extrahepatic tissues. In agreement with the findings of Ito *et al.* (1994), the liver seems to be the most sensitive organ to CYP induction, but some peculiar differences between liver, kidney and lung inducibility have been observed. The reason for differences in CYP induction in various tissues is not clear, but probably reflects the different inductive susceptibility of the CYPs in such tissues.

Hepatic metabolizing enzyme induction by Vinclozolin as well as of intestinal enzymes, was also demonstrated in

Sprague–Dawley rat and in the Japanese quail (Riviere *et al.*, 1983; Ronis *et al.*, 1994).

From a toxicological point of view, probably the greatest concern with enzyme induction is the possibility of enhanced risk of cancer. It is well-established that bioactivation is a key step in chemical carcinogenesis, and that CYP enzymes play a key role in this respect.

Altered enzyme expression may be related to variation in kinetic factors that affect tissue dosimetry, leading to cotoxicity, comutagenicity and cocarcinogenicity (IARC, 1991). Our previous study with the fungicide Fenarimol showed that perturbation of drug-metabolizing enzymes by Fenarimol enhanced the clastogenic effects of trichloroethylene in mice (Hrelia *et al.*, 1994b). Enhancement effects by Fenarimol were correlated with a significant induction of CYP2B1 and increased presence of activated trichloroethylene metabolites *in vivo*. The increased 2E1 and 3A isoforms by Vinclozolin, in addition to the alteration of endogenous metabolism where these catalysts are physiologically involved, can exert a booster phenomenon towards the bioactivation of ubiquitous environmental pollutants.

Moreover, CYP inducers may act as promoting agents in the chemical carcinogenesis process, at least concerning CYP 2B1, 1A2 and 2E1 isoforms, by either pleiotropic response (Lubet *et al.*, 1992), or oxygen centred radical overproduction (Bondy and Naderi, 1994). This increased production of reactive oxygen radicals paralleling CYP induction can impose a prooxidant status which points toward cancer (Cerutti, 1985; Bast, 1986).

However, it requires >750 mg/kg/day Vinclozolin to produce significant enzyme induction. The possibility that tumours produced by Vinclozolin in some tissues of some strains of some rodent species may arise from a secondary mechanism, such as organospecific toxicity and resultant induced cell division, if maintained at an appropriate level and for an appropriate period (Clayson, 1989; Butterworth, 1990), cannot be excluded.

In conclusion, the results presented in this study provide valuable information to achieving a more complete and accurate evaluation of the genotoxic/carcinogen profile of Vinclozolin.

Toxicity data obtained should be taken into account for the assessment of the toxicological consequences of Vinclozolin in human population potentially exposed. This is particularly true for CYP 3A and 2E1 changes, which can alter the metabolism of coadministered drugs, as well as xenobiotics biotransformed specifically by the 3A (glucocorticoids, macrolides) and 2E1 (alcohol, acetone) isozymes. This type of toxic interaction between fungicides and other chemicals might also produce toxic responses in inadvertently exposed wild life (Ronis *et al.*, 1994).

However, considering the high doses at which the fungicide showed toxicological effects in rodents, and the permissible concentrations to which humans may be exposed (ADI 0.025 mg/kg/day; EPA, 1985), the carcinogenic risk posed by Vinclozolin to humans seems to be minimal.

Acknowledgements

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INTRODUCTION

In the investigation of possible environmentally derived influences on human cancer, animal experimentation has established that a vast array of compounds are capable of playing a role in tumorigenesis, and it is now recognized that the detection and appropriate regulation of such compounds is of prime importance for the prevention of neoplasia in humans.

Such evaluations depend mainly on lifetime carcinogenicity studies in rodents. Since such tests are extremely expensive and time consuming, several *in vitro* short-term assays including the *Salmonella* mutation test have been applied as mass screening methods for detecting potentially carcinogenic compounds [1, 2]. However, there is increasing evidence to indicate that mutagenicity results do not always correlate with carcinogenicity (*Salmonella*/microsome assays, so-called Ames test) [3–5], although they are very rapid and inexpensive. The existence of discrepancies dictates the necessity of a suitable *in vivo* assay system which could bridge the gap between mutagenicity and conventional long-term carcinogenicity testing [6].

In our laboratory, a new medium-term bioassay system of only 8 weeks' duration utilizing rat liver glutathione *S*-transferase placental form (GST-P) positive foci as endpoint markers has been introduced [7–10]. The liver is an obvious choice as best organ with which to assess carcinogenicity in bioassay systems since the process of liver carcinogenesis has been well studied [11, 12], and furthermore, more than half of known carcinogens show carcinogenicity in the liver [3, 4]. Using this system, we have examined a variety of chemicals. For each tested chemical, mutagenicity and carcinogenicity derived from published data [2, 4, 5, 13–17] are also cited.

II. MATERIALS AND METHODS

A. Treatment of Animals

Figure 1 shows the protocol now employed in our laboratory for medium-term bioassays. Male F344 rats, 6 weeks old at the commencement, are divided into three groups; group 1 is given a single intraperitoneal injection of diethylnitrosamine (DEN, 200 mg/kg body weight) dissolved in saline to initiate hepatocarcinogenesis and after a 2-week recovery period on basal diet, receives one of the test compounds in the basal diet; drinking water; or by intraperitoneal, intravenous, or intragastric injections. Animals are subjected to two-thirds partial hepatectomy (PH) at week 3 to maximize any interaction between proliferation and the modification effects of the

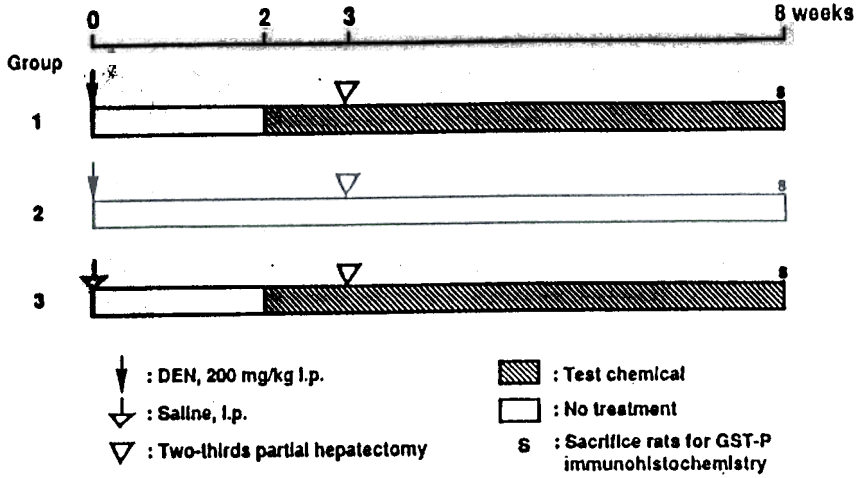

FIG. 1. Experimental protocol of the liver medium-term bioassay for carcinogenic potential.

compounds tested. Group 2 is given DEN and PH in the same manner as for group 1, without administration of any test compound. Group 3 rats are injected with saline instead of DEN solution and then subjected to test compound and PH. Doses of test compounds are chosen on the basis of preliminary investigations as permitting >70% survival of rats after PH performed during the administration, or from chronic toxicity data when available.

All animals are sacrificed at week 8, and immediately upon killing, 3 or 4 slices from the right lateral and caudate lobes are fixed in ice-cold acetone for immunohistochemical examination of glutathione *S*-transferase placental form (GST-P). Additional slices for several cases are fixed in 10% phosphate-buffered formalin solution for routine staining with hematoxylin and eosin.

B. Tissue Processing and GST-P Positive Foci Analysis

GST-P immunohistochemistry was performed as previously described [7]. Figure 2 shows GST-P-positive hepatic cell foci. The numbers and the areas of these foci larger than 0.2 mm in diameter, and the total areas of the liver sections examined were measured using a video image processor (VIP-21C, Olympus Co., Tokyo) as previously reported [7]. The results, expressed as numbers and areas of foci per unit area of liver section, were assessed by comparing the values between group 1 (DEN–test compounds)

DRUG METABOLISM REVIEWS, 26(1&2), 431–442 (1994)

MEDIUM-TERM RAT LIVER BIOASSAY FOR RAPID DETECTION OF CARCINOGENS AND MODIFIERS OF HEPATOCARCINOGENESIS

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Key Words: GST-P positive foci; Hepatocarcinogenesis; Medium-term liver bioassay; Rat; Screening of carcinogens.

*Contributed in honor of Elizabeth C. Miller, Ph.D., and James A. Miller, Ph.D.

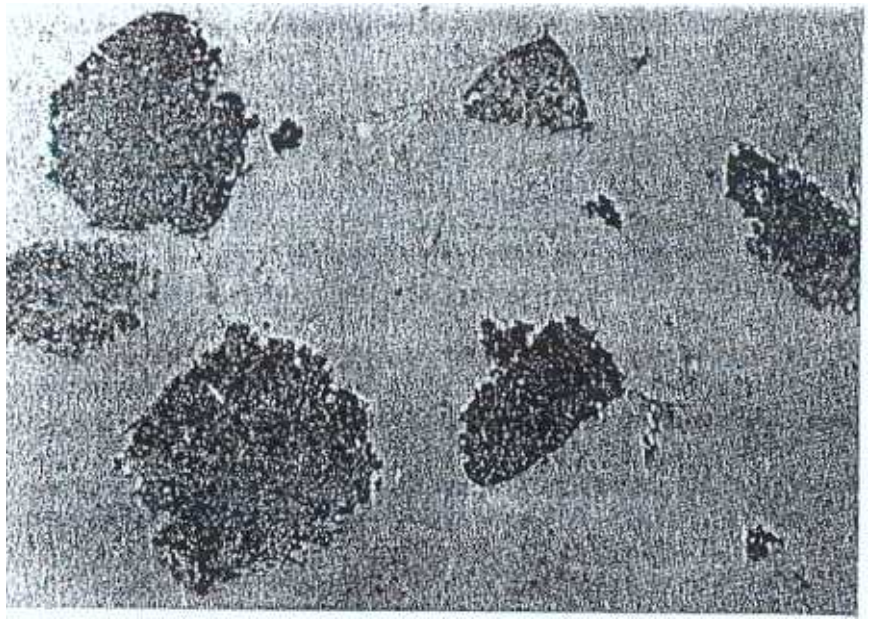

FIG. 2. GST-P-positive liver cell foci.

and group 2 (DEN alone) [8, 10]. Group 3 animals served to assay the potential of the test chemicals to induce GST-P-positive foci without prior DEN.

Statistical analysis was carried out using the Student t test and the Welch t test in combination with the F test for variability. Scoring of the results was made on the basis of the differences in quantitative values between groups 1 and 2: Positive = increase at $P < 0.05$ in either number or area of foci.

III. RESULTS AND DISCUSSION

Positive rates for the 237 compounds or mixtures so far examined are summarized in Table 1. The results are compared with Ames test and carcinogenicity data. A part of the results have been previously published [8, 10].

Of the 237 compounds, 96 exerted positive effects on GST-P-positive foci development, most of them being liver carcinogens (51 chemicals). In addition to four well-known hepatocarcinogenic peroxisome proliferators,

TABLE 1

Positive Rates in the Medium-Term Liver Bioassay

Carcinogenicity	Ames test +		Ames test −		Ambiguous		Total	
Hepatocarcinogens	28/29	(97)[a]	23/27	(86)[b]	0/1	(0)[c]	51/57	(89)
Nonhepatocarcinogens	7/25	(28)	3/14	(21)	0/2	(0)	10/41	(24)
Noncarcinogens	0/6	(0)	2/32	(6)[d]	0/2	(0)	2/40	(5)
Unknown	3/13	(23)	22/60	(37)	8/26	(31)	33/99	(33)
Total	**38/73**	**(52)**	**50/133**	**(38)**	**8/31**	**(26)**	**96/237**	**(41)**

Note. Numbers in parantheses are percentages of positive cases.

[a]4,4′-Diaminodiphenylmethane was negative.

[b]Clofibrate, di(2-ethylhexyl)phthalate, di(2-ethylhexyl)adipate, and trichloroacetic acid were negative.

[c]Dehydroepiandrosterone was negative.

[d]Malathion and vinclozolin were positive.

clofibrate, di(2-ethylhexyl)phthalate, di(2-ethylhexyl)adipate and trichloroacetic acid, the bile duct proliferator 4,4′-diaminodiphenylmethane [18], and dehydroepiandrosterone [19] were negative although these has been categorized as liver carcinogens (false negative). Dehydroepiandrosterone is known to possess peroxisome proliferating potency. With regard to the carcinogenicity of peroxisome proliferators, it is important to remember that they depress GST-P expression and that the lesions associated with their hepatocarcinogenicity are not positive for this marker enzyme [20, 21]. Some other approach to their reliable detection is clearly required.

The positive results for malathion and vinclozolin are interesting since these two chemicals have been evaluated as "noncarcinogens" in both rats and mice. These were our first cases of so-called false positives in this system [10, 22]. Malathion has been reported as "noncarcinogenic" in NTP Technical Reports [23], but liver carcinogenicity might have been suspected from the mouse results. Vinclozolin has also been reported to be noncarcinogenic by FAO/WHO [24]. However, an apparent increase in liver weight was noted for both rats and mice administered the chemical in the diet. The level of positivity is almost the same as that of phenobarbital at 0.05% in the diet; phenobarbital has been considered as a typical hepatopromoter and was recently evaluated as a liver carcinogen [3]. It may be possible that chemicals which give a positive result in this system

TABLE 2

Positively Detected Chemicals That Have Been Reported as Nonhepatocarcinogens

Carcinogen	Ames test	Principle target organs
Adriamycin	+	Kidney, mammary gland
Benzo[a]pyrene		Lung, application site
DHPN	+	Lung, thyroid
Captan	+	Duodenum
DMAB	+	Colon, prostate, pancreas
1,2-Dimethylhydrazine	+	Colon, liver?
N-Ethyl-*N*-nitrosourea	+	Nerve tissue, hematopoietic system
Alachlor		Stomach, lung, thyroid, nasal cavity
Diethylstilbestrol		Mammary gland, pituitary, liver?
D-Limonene		Kidney

Note. DHPN, *N*-bis(2-hydroxypropyl)nitrosamine; DMAB, 3,2′-dimethyl-4-aminobiphenyl.

are indeed weak hepatocarcinogens or hepatopromoters even if they have been reported as noncarcinogens or nonhepatocarcinogens in long-term animal tests.

The positive rate for carcinogens for which the liver is not included as a target (nonhepatocarcinogens) is so far only 24% (10/41); the chemicals exerting positive results are summarized in Table 2. Of the genotoxic examples in the list, *N*-bis(2-hydroxypropyl)nitrosamine (DHPN), 3,2′-dimethyl-4-aminobiphenyl (DMAB), and *N*-ethyl-*N*-nitrosourea (ENU) are known to exert carcinogenicity in a variety of organs.

Of 41 nongenotoxic carcinogens examined, 26 compounds proved positive in this system (Table 3). Many are chlorinated compounds showing carcinogenicity in the liver [4] and antioxidants [25], both of which are usually negative in the Ames test. Liver carcinogens for which carcinogenicity has been described only in mice were also detected at a high rate (11 chemicals). Among 15 nongenotoxic carcinogens which did not exert positive effects in the present system, 11 compounds are nonhepatocarcinogens. Four nongenotoxic hepatocarcinogens are peroxisome proliferators as mentioned above.

The Ames test gave higher positivity for nonhepatocarcinogens than the present model. Since the present system is based on the two-stage hypothesis of carcinogenesis, a positive result means that the test chemical basically possesses tumor-promoting activity in the liver. However, several

TABLE 3

Results for Carcinogens That Are Negative in the Ames Test

Hepatocarcinogens	Nonhepatocarcinogens
Positive	Positive
Aldrin[a,c]	Alachlor[a]
Auramine O	Diethylstilbestrol
Barbital[c]	*D*-Limonene
Chlordane[a,c]	
Chlorendic acid[a,c]	
Chlorobenzilate[a]	Negative
p,p′-DDT[a]	Acetaldehyde
Dieldrin[a,c]	Atrazine[a]
Ethenzamide[c]	Daminozide
17α-Ethinyl estradiol	1,1-Dimethylhydrazine
Ethionine	Propineb
Hexachlorobenzene[a]	Sodium *O*-phenylphenate
α-Hexachlorocyclohexane[c]	Sodium saccharin
Phenobarbital	Butylated hydroxyanisole
Prochloraz[a,c]	Catechol
Propiconazole[a,c]	Hydroquinone
Safrole	Sesamol
Sulpyrin[c]	
Tannic acid	
Thioacetamide	
Triadimefon[a,c]	
Trifluralin[c]	
Urethane	
Negative	
Di(2-ethylhexyl)adipate[b,c]	
Trichloroacetic acid[b,c]	
Clofibrate[b]	
Di(2-ethylhexyl)phthalate[b]	
Rate 23/27 (86%)	3/14 (21%)

[a]Chlorinated.
[b]Peroxisome proliferator.
[c]Carcinogenicity reported only in the mouse liver.

TABLE 4

Chemicals That Demonstrated Inhibition in This Model

Compound	Ames test	Compound	Ames test
Carcinogens (8 chemicals)			
AF-2	+		+
Butylated hydroxyanisole	−		+
Catechol			+
Clofibrate			
Di(2-ethylhexyl)phthalate			
Hydroquinone	−		
Sesamol	−		−
Caffeic acid	?		−
Noncarcinogens (6 chemicals)			
Acetaminophen			
Benzoin			
Gallic acid			−
α-Tocopherol	−		?
DEF[a]	?		?
Esculin	?		?
			?

[a]DEF, *S,S,S*-tributylphosphorotrithioate.

nonhepatocarcinogens were also detected by this model. It may, of course, be possible that chemicals reported as noncarcinogens or nonhepatocarcinogens in long-term animal tests which gave a positive result in this system are indeed weak hepatocarcinogens or hepatopromoters.

The model could also be useful for examination of chemopreventive agents [26, 27]. Chemicals which showed inhibitory effect in this system are summarized in Table 4. Of the 31 examples, carcinogenic activity has been demonstrated for 8 chemicals, and 6 chemicals have been reported as noncarcinogenic. The system is applicable for investigation of chemopreventive activity and also can be modified so that the candidate chemical is administered with a known carcinogen during the 6-week treatment period to evaluate the effects of simultaneous exposure.

In conclusion, the liver system described here is proposed as a practical tool for the rapid detection of carcinogenic potential of environmental compounds. The vast majority of liver carcinogens proved positive, although the detection rate for carcinogens targeting tissues other than the liver is not as satisfactory. Such carcinogens could, however, be detected using whole-body systems, and we have proposed several models for that purpose [28–30] based on induction of preneoplastic lesions [31]. The future should bring a new general scheme for guidelines in the screening of carcinogenic agents.

IV. SUMMARY

For rapid detection of carcinogenic agents, a medium-term liver bioassay has been established in our laboratory using preneoplastic glutathione *S*-transferase placental form (GST-P) positive foci in the rat liver as endpoint marker lesions. A total of 237 compounds have so far been tested in this system and the results compared with reported *Salmonella*/microsome and long-term carcinogenicity test findings. The positive rate was found to be extremely high, 97% (28 of 29 compounds) for genotoxic hepatocarcinogens; and satisfactory, 86% (23 of 27 chemicals) for nongenotoxic ones. The positive rate for carcinogens targeting organs other than the liver, however, is relatively low (24%). Malathion and vinclozolin proved positive, although both have been reported to be noncarcinogenic in rats and mice. Those chemicals which exerted positive results in this system might be hepatopromoting agents even if hepatocarcinogenicity has not been established. Five of the six false-negative hepatocarcinogens could be categorized as peroxisome proliferators. In addition, a number of inhibitory agents for GST-P-positive foci development have been detected and many are categorized as antioxidants. The validity of this system as a tool for rapid detection of carcinogenic and chemopreventive agents is discussed.

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POLYCYCLIC HYDROCARBON ACTIVATION: BAY REGIONS AND BEYOND*

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Genotoxicity of selected pesticides in the mouse bone-marrow micronucleus test and in the sister-chromatid exchange test with human lymphocytes in vitro

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Abstract

Selected pesticides (aldicarb, 1,3-dichloropropene, methidathion, parathion, triadimefon, vinclozolin) were tested for their clastogenic and aneugenic activities in the mouse bone-marrow micronucleus (MN) test in vivo and for their sister-chromatid exchange-inducing activities in human lymphocytes in vitro in the presence and absence of an exogenous metabolizing system from rat-liver S9. 1,3-Dichloropropene significantly increased the frequencies of micronucleated polychromatic erythrocytes (PCE) in bone-marrow cells of female mice from 3.3 MN/1000 PCE to 15.3 MN/1000 PCE (187 mg per kg body weight). 1,3-Dichloropropene (100 μM) induced 16.0 SCE/metaphase after 24 h of incubation as compared with the basal rate of 11.2 SCE/metaphase (− S9) and of 15.4 SCE/metaphase as compared with 10.5 SCE/metaphase of the control (+ S9). These values were statistically significantly different from each other. The other pesticides tested did neither increase the rate of micronuclei significantly in polychromatic erythrocytes in male nor in female animals. Aldicarb and methidathion induced a significant increase in SCEs in human lymphocytes in vitro only without the metabolic activating system: aldicarb, 5 μM, 24 h incubation: 15.5 SCE/metaphase; control: 12.6 SCE/metaphase; methidathion, 100 μM, 24 h incubation: 15.8 SCE/metaphase, control: 11.1 SCE/metaphase. Parathion, triadimefon and vinclozolin did not have any SCE-inducing effects.

Keywords: Micronucleus test (MNT) in vivo; Bone-marrow; Sister-chromatid exchange (SCE) in vitro; Human lymphocytes; Genotoxicity; Pesticides

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1. Introduction

Vast amounts of pesticides are used for agricultural applications worldwide each year. Therefore thorough studies of these xenobiotics are essential in particular their effects on environmental systems and human health.

In the present study selected pesticides of different chemical classes were tested for their clastogenic and aneugenic activities in the mouse bone-marrow micronucleus test (MNT) in vivo and for their sister-chromatid exchange (SCE) inducing activities in human lymphocytes. Aldicarb and methidathion are acetylcholine esterase inhibitors and are used as insecticides, nematocides and acaricides. 1,3-Dichloropropene consists of *cis*- and *trans*-isomers (1:1) and was used as soil nematocide in vegetable- and tobacco-growing agriculture. Parathion is an organophosphate ester and is metabolized in the organism via oxidation and hydrolysis. Triadimefon belongs to the chemical group of triazol derivatives and vinclozolin to the oxazolidin derivatives, both are fungicides.

2. Materials and methods

Pesticides tested were obtained from Promochem (Wesel, Germany; purity: aldicarb 99.9%, 1,3-dichloropropene 95.0%, methidathion 99.9%, parathion 98.6%, triadimefon 99.9%, vinclozolin 99.9%).

2.1. In vivo micronucleus test

In vivo micronucleus test was performed as follows [1,2]. Each pesticide was tested in male mice in four doses approximating 50, 80, 100 and 115% of published LD_{50} values [3–5] and subsequently in female mice in two doses corresponding to 80 and 100% of these LD_{50} values. By doing so, the LD_{50} was used to define a maximum tolerated dose [2]. The lower number of treatment schedules with female mice was chosen on account of the negative results obtained with male mice in order to save animals. Each dose was applied to four female and four male NMRI mice. NMRI mice, aged 7–12 weeks, body weight 25–35 g, were supplied by Interfauna (Süddeutsche Versuchstierfarm, Tuttlingen). In each experiment cyclophosphamide (male: 600 mg/kg body weight; female: 450 mg/kg body weight) was used as positive control. The pesticides were dissolved in 200 or 300 μl corn oil ('Mazola', Maizena, Heilbronn) and administered to the animals by oral gavage. As negative control only corn oil was administered. Because of the high concentration tested, vinclozolin, however, had to be applied in six portions (each 300 μl of corn oil as solvent) with an intermission of 1 h between each application.

The animals were caged in groups by sex. Appropriate diet and drinking water was supplied ad libitum. Temperature (22° ± 2°C), relative humidity (55% ± 5%) and day-night light cycles of 12 h each were controlled as dictated by good animal husbandry procedures. After 48 h the animals were gassed with CO_2, killed by cervical dislocation and the femurs were excised. A sampling time of 48 h seemed to be more advantageous than 24 h on account of findings by Vanparys et al. [6], who reported higher numbers of micronucleated polychromatic erythrocytes at that time for seven of eight standard compounds. Using 5 ml fetal calf serum the bone marrow from both femurs was centrifuged for 5 min at 200 × *g*. The supernatant was discarded, leaving roughly 100 μl in which the pellet was resuspended carefully. One drop of this suspension was spread on a slide of a refrigerated glass top. Slides were prepared in triplicate for each animal. The slides were air-dried for 12 h, then staining was performed as follows: first, the slides were stained for 3 min in May-Grünwald solution, then for 2 min in a solution of May-Grünwald/demineralized water 1:1 (v/v). Afterwards the slides were washed with demineralized water, stained in Giemsa solution for 10 min and washed again. After air-drying for half an hour, 2 drops of Entellan (Merck, Darmstadt, FRG) were added. Then, 24 h later after further air-drying the slides were prepared for analysis.

These slides were coded for blind analysis. At least 1000 polychromatic erythrocytes per animal were scored for the presence of micronuclei. The ratio of polychromatic to normochromatic ery-

throcytes was also determined by counting a total of 1000 erythrocytes. Mean values and standard deviations were calculated. To evaluate the statistical significance of the results, the Mann-Whitney-Wilcoxon test was used.

2.2. Sister-chromatid exchange test

The sister-chromatid exchange test in vitro was performed as follows [7–9]. For the human lymphocyte in vitro sister-chromatid exchange (SCE)-test cultures were set up from fresh human blood, collected aseptically by venipuncture from healthy non-smoking donors aged from 25–35 years. Each culture consisted of 0.3 ml blood in 5.0 ml chromosome-medium (Chromosomen-medium B PHA-M from Biochrom, Berlin FRG). 5-Bromo-2‰-deoxyuridine (Serva, Heidelberg, FRG) was added to each culture at a concentration of 6 μg/ml.

Cultures were treated with various concentrations of each test compound for 24 and for 48 h in the absence of a metabolic activating system. In a third experiment lymphocytes were treated in the presence of rat-liver S9-Aroclor 1254 (Organon Technika, Eppelheim, FRG) for 2 h (0.37 or 3.7 mg total protein content/ml culture medium). In the case of metabolic activation rat-liver S9 was added 48 h after the start of culturing. Pesticides were tested in concentrations up to a cytotoxic response in the culture determined by an absence of dividing cells. All pesticides tested were dissolved in DMSO, pure DMSO was used as negative control.

In total, lymphocytes were cultivated for 72 h at 37°C. Two h before termination colcemid (0.4 μg/ml) was added. As positive control cyclophosphamide was used at a concentration of 10^{-5} mol/l with and without S9. The SCE frequencies induced by cyclophosphamide were in the range of 70 and 85 SCE per metaphase in the presence and between 8.9 and 10.5 in the absence of S9. The cells were harvested and slides were prepared according to the method of Perry and Wolff [7] for the analysis of sister-chromatid exchanges. One hundred metaphases were scored to determine the cell-proliferation. Each metaphase was classified as being in the first (M1), second (M2), and third (M3) division. The proliferation index (PRI) was calculated as follows: $PRI = (1 \times M1 + 2 \times M2 + 3 \times M3)/100$ [10]. Furthermore, 30 metaphases were examined for sister-chromatid exchanges for each concentration of the test-compounds and the controls.

3. Results and discussion

As can be seen from Table 1, none of the pesticides aldicarb, methidathion, parathion, triadimefon and vinclozolin increased the rate of micronuclei in polychromatic erythrocytes significantly neither in male nor in female animals in the vivo mouse bone-marrow micronucleus test (MNT). In contrast, 1,3-dichloropropene yielded a significant increase in the frequency of micronuclei (MN) in female mice, shown more detailed in Table 2. Surprisingly, nearly all means of MN/1000 PCE as well as the PCE/NCE ratios were higher in female animals as compared with male ones. We have no explanation for these observations, though differences in background micronucleus frequencies between males and females have been reported for many common laboratory strains of mice [2]. We have no reason to assume that there is a technical cause for the differences observed.

A significant increase (Mann-Whitney-Wilcoxon test, $P < 0.01$) with a mean of 15.3 micronuclei per 10^3 polychromatic erythrocytes was caused in female mice after application of 187 mg 1,3-dichloropropene per kg body weight. A slight decrease in the number of micronuclei (mean 14.9 MN per 10^3 polychromatic erythrocytes) was caused by 234 mg 1,3-dichloropropene per kg body weight. Some authors proposed to use male mice exclusively for micronucleus testing of chemicals on account of an assumed higher sensitivity [11,12]. Analysis of available experimental data with positive results, however, presented separately for male and female animals, showed little or no sex differences for about two-thirds of compounds tested but a greater micronucleus response of several substances in males or females with no preference of either sex [2]. Our results with an unique response of female mice

Table 1
Frequencies of micronucleated polychromatic erythrocytes in bone-marrow cells of NMRI mice after pesticide treatment

Pesticide	Highest tolerated dose (mg/kg b.w.)[a]	Sex	MN/10³ PCE ± S.D.	PCE/NCE ± S.D.
Aldicarb	0.9	Male	4.00 ± 1.83[b]	0.51 ± 0.09
	0.47	Female	1.56 ± 0.72	1.35 ± 0.15
,3-Dichloropropene	280	Male	0.40 ± 0.42	0.64 ± 0.18
	234	Female	14.9 ± 1.45[c]	1.85 ± 0.18
Methidathion	27.5	Male	1.13 ± 1.11	0.85 ± 0.05
	25	Female	2.31 ± 0.55	1.54 ± 0.17
Triadimefon	1200	Male	1.63 ± 1.25	0.43 ± 0.10
	1071	Female	4.56 ± 0.92	1.18 ± 0.13
Parathion	2.2	Male	1.50 ± 2.00	0.76 ± 0.29
	1.5	Female	3.56 ± 0.85	1.55 ± 0.22
Vinclozolin	1150	Male	2.00 ± 0.73	0.73 ± 0.19
	1000	Female	5.44 ± 1.60	1.41 ± 0.15
Control ($n = 12$)	Corn oil	Male	0.83 ± 1,50	0.76 ± 0.39
	Corn oil	Female	2.81 ± 1,30	1.60 ± 0.24

[a]Mice were tested in the highest tolerated dose, i.e. when all animals survived.
[b]Mean frequencies of micronuclei of four animals and the standard deviations are given.
MN, micronuclei; PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes; S.D., standard deviation; b.w., body weight; n, number of animals. In each group a positive control with cyclophosphamide (female mice: 450 mg/kg b.w.; male mice: 600 mg/kg b.w.) was performed.
[c]Statistically significant in the Mann-Whitney-Wilcoxon test ($P < 0.01$).

with respect to 1,3-dichloropropene support the concept of using male as well as female animals to check for micronuclei-inducing pesticides. Otherwise positive results may be missed.

In vitro 1,3-dichloropropene induced increases in SCE-frequencies in human lymphocytes with and also without addition of an exogenous metabolizing system ($P < 0.01$, one-sided t-test) (Table 3). The assay with 24 h incubation time showed a mean induction of 11.2 SCE/metaphase up to 16.0 SCE/metaphase for the highest 1,3-dichloropropene concentration (100 μM). After 48 h incubation time, the mean of 9.4 SCE/metaphase increased to up to 23.0 SCE/metaphase in the highest concentration tested (100 μM). In the presence of an exogenous metabolic activating system (rat liver S9), however, the mean basal SCE rate of 10.5 was elevated up to 15.4 after application of 100 μM 1,3-dichloropropene.

1,3-Dichloropropene (purity 95%) may contain a number of impurities, such as 1,2-dichloropropane, that was mutagenic in *Salmonella* [13] and induced chromosome aberrations [14]. Pure 1,3-dichloropropene is instable and therefore normally stabilized, stabilizers may be genotoxic. Two oxidation products of 1,3-dichloropropene, epichlorohydrin and 1,3-dichloro-2-propanol are known mutagens [15]. 1,3-Dichloropropene of high purity was shown to be mutagenic in *Salmonella* only with metabolic activation [16–18]. Trade products with 1,3-dichloropropene were mutagenic in *Salmonella* with and without metabolic activation because of impurities the technical product contains [13,15,18]. 1,3-Dichloropropene induced squamous cell carcinoma and papilloma in mice [19]. In 1985 the U.S. EPA classified 1,3-dichloropropene as a substance with carcinogenic potential [20].

With 24 h incubation time, aldicarb showed a mean of 12.6 SCE/metaphase up to 15.5 SCE/metaphase for the highest concentration of the test-compound (5 μM) ($P < 0.05$, one-sided t-test). After 48 h incubation time there was a mean increase of 10.8 SCE/metaphase up to 14.0 SCE/metaphase for the highest concentration of the test-compound (5 μM) ($P < 0.01$, one-sided t-test). These effects were only ascertained in cultures without the metabolic activating system,

Table 2
Induction of micronucleated polychromatic erythrocytes in bone-marrow cells in NMRI mice after treatment with 1,3-dichloropropene

Dose (mg/kg b.w.)	Number of animals	MN/10^3 PCE ± S.D.	PCE/NCE ± S.D.
Male mice			
0		2.0	0.39
600 CP (pos. control)		85.0	0.10
140	4	1.60 ± 0.14[a]	0.42 ± 0.10
280	4	0.40 ± 0.42	0.64 ± 0.18
Female mice			
0		3.3	1.35
450 CP (pos. control)	1	75.8	0.82
187	4	15.3 ± 1.88[c]	1.56 ± 0.19
234	4/3[b]	14.9 ± 1.45[c]	1.85 ± 0.18

[a]Mean and standard deviation.
[b]Number of animals that survived.
CP, cyclophosphamide; MN, micronuclei; PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes; b.w., body weight.
[c]Mann-Whitney-Wilcoxon test ($P < 0.01$).

whereas in the presence of rat-liver S9, no genotoxic response could be detected (Table 3).

Aldicarb was neither mutagenic in *Salmonella* nor in *Escherichia coli* WP2 uvrA [21,22], but it induced chromosomal aberrations in human lymphocytes [23]. There were no indications for a carcinogenicity of aldicarb [24–26].

Methidathion induced significant increases ($P < 0.01$, one-sided t-test) of SCE-frequencies in human lymphocytes in vitro only in the absence of rat liver S9 (Table 3). For an incubation time of 24 h, the mean of 11.1 SCE/metaphase increased to 15.8 SCE/metaphase in the highest concentration of the test-compound (100 μM). After 48 h a mean rise of 10.6 SCE/metaphase up to 15.4 SCE/metaphase for the highest concentration of the test-compound (100 μM) was detected. These results were confirmed by investigations with the cell line V79 Chinese hamster lung [27]. Methidathion occured to be negative in *Salmonella* and in the MNT in vivo in our own investigations ([28]; Table 1).

Triadimefon and parathion were shown to be not mutagenic in *Salmonella* and *Escherichia coli* WP2 [29,30]. These substances did not induce SCEs and they were negative in the recombination test with *Saccharomyces cerevisiae* D3 [29–31]. Our own investigations did not show any evidence for genotoxicity of triadimefon and parathion neither in the *Salmonella* test nor in the sister-chromatid exchange assay [28]. Triadimefon as well as parathion did not induce micronuclei in NMRI mice (Table 1). Maronpot et al. tested parathion in a tumor-induction-bioassay with mice [32]. A conceivable carcinogenicity of parathion was indicated by a significant increase ($P < 0.05$) of pulmonary tumors in mice. Data concerning the carcinogenicity of triadimefon and parathion are not available to date.

In our own investigations, vinclozolin did not show any effects in the MNT in vivo (Table 1), the SCE in vitro and in *Salmonella* [28]. Vinclozolin neither was genotoxic in the DNA-repair test with *Bacillus subtilis* M17 and M45 nor in the mouse lymphoma test nor in the HGPRT test with CHO cells [33]. Two studies concerning the carcinogenicity of vinclozolin did not show any effects in NMRI mice and Sprague–Dawley rats [33].

Table 3
Induction of sister chromatid exchanges (SCEs) in cultured human lymphocytes by aldicarb, 1,3-dichloropropene, methidathion, triadimefon, parathion and vinclozolin

S9	Incubation time (h)	Concentration (μmol/l)			
Aldicarb		0	0.5		
	24	12.6 ± 4.7[a]	11.4 ± 5.6	12.5 ± 5.1	15.5 ± 5.7*
		1.8[b]	1.8	1.7	1.9
	48	10.8 ± 4.3	13.1 ± 5.0	12.8 ± 5.3	14.0 ± 5.3**
		2.0	1.9	1.7	2.1
0.1[c]		12.0 ± 4.9	15.0 ± 4.7	12.6 ± 5.5	12.7 ± 4.7
		2.0	2.0	2.0	1.9
	2	8.9 ± 3.5	n.d.	9.8 ± 2.6	9.8 ± 3.9
		1.3		1.8	2.1
1,3-Dichloropropene		0	1	10	100
	24	11.2 ± 4.3[a]	8.7 ± 3.7	10.1 ± 4.0	16.0 ± 5.3**
		1.8[b]	2.2	2.1	1.6
	48	9.4 ± 3.7	9.1 ± 4.3	9.4 ± 3.5	23.0 ± 7.0**
		1.8	2.1	2.0	2.1
		10.5 ± 4.3	10.0 ± 4.:	9.1 ± 3.6	15.4 ± 4.3**
		2.0	1.7	1.8	1.5
Methidathion		0		10	100
	24	11.1 ± 4.8[a]	n.d.	10.5 ± 4.9	15.8 ± 6.0 * *
		2.0[b]		2.0	2.8
	48	10.6 ± 4.5	8.5 ± 3.6	12.4 ± 5.2	15.4 ± 5.7 * *
		2.1	1.9	2.2	2.0
1[c]		11.4 ± 4.7	n.d.	10.5 ± 3.6	12.4 ± 3.6
		1.7		1.9	1.9
Triadimefon		0	50	100	–
	24	11.9 ± 5.6[a]	n.d.	11.1 ± 5.7	
		1.9[b]		2.1	
	48	12.4 ± 5.5	11.8 ± 4.7	12.8 ± 6.3	
		2.1	1.9	1.8	
	2	10.1 ± 6.0	9.0 ± 4.0	n.d.	
		2.0	2.0		
Parathion		0	1	10	100
	24	11.2 ± 4.3[a]	n.d.	10.0 ± 3.9	11.2 ± 3.6
		1.3[b]		1.5	1.3
	48	9.4 ± 3.7	8.8 ± 3.	11.0 ± 3.6	n.d.
		1.8	0.6	2.0	
		10.5 ± 4.3	n.d.	10.1 ± 3.8	9.9 ± 4.4
		1.4		1.4	1.4
Vinclozolin		0	10	100	–
	24	8.9 ± 3.4[a]	8.8 ± 3.3	9.3 ± 2.7	
		2.1[b]	2.2	1.5	
	48	9.1 ± 3.5	9.2 ± 3.4	8.7 ± 3.8	
		2.2	2.3	1.9	
	2	9.1 ± 3.4	11.5 ± 3.1	10.9 ± 4.2	
		2.2	2.1	1.8	

Number of samples examined were as described in Materials and methods (Section 2). [a]Mean and standard deviation. [b]Proliferation index. [c]mg protein per ml culture. n.d., not determined. *$P<0.05$ (one-sided t-test); **$P<0.01$ (one-sided t-test).

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Screening for Estrogen and Androgen Receptor Activities in 200 Pesticides by *In Vitro* Reporter Gene Assays Using Chinese Hamster Ovary Cells

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We tested 200 pesticides, including some of their isomers and metabolites, for agonism and antagonism to two human estrogen receptor (hER) subtypes, hERα and hERβ, and a human androgen receptor (hAR) by highly sensitive transactivation assays using Chinese hamster ovary cells. The test compounds were classified into nine groups: organochlorines, diphenyl ethers, organophosphorus pesticides, pyrethroids, carbamates, acid amides, triazines, ureas, and others. These pesticides were tested at concentrations < 10^{-5} M. Of the 200 pesticides tested, 47 and 33 showed hERα- and hERβ-mediated estrogenic activities, respectively. Among them, 29 pesticides had both hERα and hERβ agonistic activities, and the effects of the organochlorine insecticides β-benzene hexachloride (BHC) and δ-BHC and the carbamate insecticide methiocarb were predominantly hERβ rather than hERα agonistic. Weak antagonistic effects toward hERα and hERβ were shown in five and two pesticides, respectively. On the other hand, none of tested pesticides showed hAR-mediated androgenic activity, but 66 of 200 pesticides exhibited inhibitory activity against the transcriptional activity induced by 5α-dihydrotestosterone. In particular, the antiandrogenic activities of two diphenyl ether herbicides, chlornitrofen and chlomethoxyfen, were higher than those of vinclozolin and *p,p′*-dichlorodiphenyl dichloroethylene, known AR antagonists. The results of our ER and AR assays show that 34 pesticides possessed both estrogenic and antiandrogenic activities, indicating pleiotropic effects on hER and hAR. We also discussed chemical structures related to these activities. Taken together, our findings suggest that a variety of pesticides have estrogenic and/or antiandrogenic potential via ER and/or AR, and that numerous other manmade chemicals may also possess such estrogenic and antiandrogenic activities. *Key words:* antiandrogenic activity, Chinese hamster ovary cells, estrogenic activity, human androgen receptor, human estrogen receptor α, human estrogen receptor β, pesticide, reporter gene assay. *Environ Health Perspect* 112:524–531 (2004). doi:10.1289/ehp.6649 available via *http://dx.doi.org/* [Online 3 December 2003]

It has been well documented that several chemicals from agricultural, industrial, and household sources possess endocrine-disrupting properties, which provide a potential threat to human and wildlife reproduction (Colborn 1995; Colborn et al. 1993; Jensen et al. 1995). A suggested mechanism is that these environmental contaminants alter the normal functioning of the endocrine and reproductive system by mimicking or inhibiting endogenous hormone action, modulating the production of endogenous hormones, or altering hormone receptor populations (Sonnenschein and Soto 1998). A major mechanism of endocrine disruption is the action of chemicals as receptor agonists or antagonists through direct interaction with hormone receptors, thus altering endocrine function. In particular, chemicals mimicking endogenous estrogen via estrogen receptor (ER) have been the focus of research for the last 20 years. Meanwhile, recent studies have shown that several chemicals may exert antiandrogenic effect by interfering with androgen receptor (AR; Sohoni and Sumpter 1998; Vinggaard et al. 1999).

Pesticides commonly used to control agricultural and indoor pests are the most likely suspects as endocrine disruptors. The ubiquitous nature of pesticide usage with minimal precautions has resulted in contamination of food, the workplace, and the environment. Recent reports showed that several pesticides exert estrogenic and antiandrogenic activities through interaction with estrogen and androgen receptors. To date, *p,p′*-dichlorodiphenyl trichloroethane (DDT) (Welch et al. 1969), methoxychlor (Bulger et al. 1978; Cummings 1997), β-benzene hexachloride (BHC) (Coosen and Velsen 1989), endosulfan, toxaphene, and dieldrin (Soto et al. 1995), and fenvalerate (Garey and Wolff 1998) have been reported as estrogenic pesticides. Recently, Andersen et al. (2002) have reported that several currently used pesticides, such as methiocarb, fenarimol, chlorpyrifos, deltamethrin, and tolclofos-methyl, possess estrogenic activity on the basis of cell proliferation assay and transactivation assay using MCF-7 human breast cancer cells. On the other hand, studies have also revealed antiandrogenic pesticides, such as vinclozolin and *p,p′*-dichlorodiphenyl dichloroethylene (DDE) (Kelce et al. 1994, 1995), DDT isomer and methoxychlor (Maness et al. 1998), linuron (Gray et al. 1999; Lambright et al. 2000), procymidone (Ostby et al. 1999), and fenitrothion (Tamura et al. 2001). Andersen et al. (2002) reported that dieldrin, endosulfan, methiocarb, and fenarimol possessed antiandrogenic activity on the basis of transactivation assay using Chinese hamster ovary (CHO) cells. Thus, estrogenic and antiandrogenic activities have been found in a number of pesticides, and it is conceivable that many other pesticides also have estrogenic and/or antiandrogenic activity.

Transactivation or reporter gene assay, which is a powerful tool for testing receptor agonists and antagonists among chemicals, has been established as a method for evaluating the receptor activity of chemicals. However, many compounds are independently evaluated for ER or AR activity by different reporter gene assays. This may lead to confusion in the evaluation of their potential as endocrine-disrupting chemicals. In addition, the recent cloning of a gene for a second estrogen receptor, ERβ, by Kuiper et al. (1996) led to the discovery that ERβ and the classic ERα differ in their ligand binding ability and transactivation properties (Kuiper et al. 1997; McInerney et al. 1998). Therefore, a screening system involving both ER subtypes (α and β) is required to completely evaluate the endocrine disruption potential of environmental estrogens. Previously, we reported highly sensitive reporter gene assays using CHO cells for detecting ERα and AR agonists/antagonists from chemicals, and demonstrated that the diphenyl ether–type herbicide chlornitrofen (CNP) and its amino derivatives (CNP-amino) possessed both antiandrogenic and estrogenic activities (Kojima et al. 2003). In the present study, we screened a total of 200 pesticides using our reporter gene assay systems for detecting two ER subtypes, ERα and ERβ, and AR activities. These pesticides, including several well-known estrogenic and antiandrogenic pesticides such as DDT and vinclozolin, were selected according to the frequency of their use in Japan and other countries, both currently and in the past. They are classified

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into nine groups according to similarities in their chemical structure and are discussed on the basis of the relationships between chemical structure and activity via hormone receptors. In this article, we provide evidence that a variety of pesticides have estrogenic and/or antiandrogenic potential via ER and/or AR and that their activities are related to chemical structure.

Materials and Methods

Chemicals. 17β-Estradiol (E_2; > 97% pure), 5α-dihydrotestosterone (DHT; 95% pure), and tamoxifen citrate (98% pure) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The 200 pesticides tested in the present study are listed in Table 1. These pesticides were purchased from Wako, Sigma-Aldrich (St. Louis, MO, USA), Dr. Ehrenstorfer GmbH (Augsburg, Germany), AccuStandard Inc. (New Haven, CT, USA), and Hayashi Pure Chemical Industries, Ltd. (Osaka, Japan) and had a purity of 95–100%. Dimethylsulfoxide (DMSO) was also purchased as a vehicle from Wako.

E_2 and DHT were stored as a 1-mM stock solution in DMSO at –20°C. Pesticides were dissolved in DMSO to a final concentration of 10 mM, except for asulam, paraquat, diquat, iminoctadine, glyphosate, and carbendazim, which were directly dissolved in the medium, and all pesticides were diluted to the desired concentrations in phenol red–free Dulbecco's modified Eagle medium plus nutrient mixture Ham's F-12 (DMEM/F-12) immediately before use. The final solvent concentration in the culture medium did not exceed 0.1%, and this concentration did not affect cell yields.

Cell line and cell culture conditions. CHO-K1 cells were obtained from the Dainippon Pharmaceutical Co. (Osaka, Japan). Penicillin–streptomycin solution (antibiotics) and DMEM/F-12 were obtained from Gibco-BRL (Rockville, MD, USA). Fetal bovine serum (FBS) and charcoal-dextran–treated (CD) FBS were obtained from Hyclone (Logan, UT, USA). For routine maintenance, cells were grown in DMEM/F-12 supplemented with 10% FBS and antibiotics at 37°C in an atmosphere of 5% CO_2/95% air under saturating humidity and passaged every week by trypsinization with 0.25% trypsin/0.02% ethylenediamine tetraacetic acid (EDTA) disodium salt solution (Life Technologies, Paisley, UK).

Construction of plasmids. The human ERα (hERα) and AR (hAR) expression vectors (pcDNAERα and pZeoSV2AR) were constructed as previously described (Kojima et al. 2003). The hERβ expression vector was newly constructed as follows: The ERβ cDNA was cloned by reverse transcriptase–polymerase chain reaction from human placental RNA (Clontech, Palo Alto, CA, USA). The sequence

Table 1. The 200 pesticides tested in the reporter gene assays for hERα, hERβ, and hAR.

Group, compound	Group, compound	Group, compound
1. Organochlorines (n = 29)	Isofenphos	Mepronil
Aldrin	Isoxathion	Metalaxyl
α-BHC	Leptophos	Metolachlor
β-BHC	Malathion	Pretilachlor
γ-BHC	Mecarbam	Propyzamide
δ-BHC	Methamidophos	Thenylchlor
Captan	Methidathion	7. Triazines (n = 7)
cis-Chlordane	Methyl-parathion	Anilazine
trans-Chlordane	Monocrotophos	Atrazine
Chlorobenzilate	Parathion	Metribuzin
Chloropropylate	Phenthoate	Prometon
Chlorothalonil	Phorate	Prometryn
o,p'-DDT	Phosalone	Simazine
p,p'-DDT	Phosmet	Simetryn
p,p'-DDE	Piperophos	8. Ureas (n = 8)
p,p'-DDD	Pirimiphos-methyl	Bensulfuron-methyl
Dichlobenil	Profenofos	Daimuron
Dicofol	Propaphos	Diflubenzuron
Dieldrin	Prothiofos	Diuron
α-Endosulfan	Prothiofos oxon	Linuron
β-Endosulfan	Pyridaphenthion	Pencycuron
Endosulfan sulfate	Quinalphos	Prochloraz
Endrin	Terbufos	Propanil
Folpet	Tetrachlorvinphos	9. Others (n = 44)
Fthalide	Thiometon	Amitraz
Heptachlor	Tolclofos-methyl	Benfuresate
Heptachlor epoxide	Tolclofos-methyl oxon	Bentazone
Methoxychlor	Trichlorfon	Benzoximate
Pentachlorophenol	Vamidothion	Biphenyl
Quintozene	4. Pyrethroids (n = 12)	Bitertanol
2. Diphenyl ethers (n = 11)	Cyfluthrin	Bromopropylate
Acifluorfen	Cyhalothrin	Chinomethionat
Acifluorfen-methyl	Cypermethrin	Chloridazon
Bifenox	Deltamethrin	Dazomet
Chlomethoxyfen	Etofenprox	Diquat
Chlomitrofen	Fenvalerate	Ethoxyquin
Chlomitrofen-amino	Flucythrinate	Fenarimol
Chloroxuron	Fluvalinate	Ferimzone
Diclofop-methyl	Permethrin	Fluazinam
Fluazifop-butyl	Pyrethrin	Imazalil
Nitrofen	Tefluthrin	Imidacloprid
Oxyfluorfen	Tralomethrin	Iminoctadine
3. Organophosphorus pesticides (n = 56)	5. Carbamates (n = 22)	Indanofan
Acephate	Bendiocarb	Ioxynil octanoate
Anilofos	Benomyl	Iprodione
Bromophos-ethyl	Carbaryl	Isoprothiolane
Bromophos-methyl	Carbendazim	Lenacil
Butamifos	Carbofuran	4-Chloro-*o*-toloxyacetic acid (MCPA)
Chlorpyrifos	Chlorpropham	2,4-Dichlorophenoxyacetic acid (2,4-D)
Chlorpyrifos-methyl	Diethofencarb	Paraquat
Cyanofenphos	Dimepiperate	Pendimethalin
Cyanophos	Esprocarb	2-Phenylphenol
Diazinon	Ethiofencarb	Probenazole
Dichlofenthion	Fenobucarb	Procymidone
Dichlorvos	Isoprocarb	Propiconazole
Dimethoate	Methiocarb	Pyrazolynate
Dioxabenzofos	Methomyl	Pyrazoxyfen
Disulfoton	Molinate	Pyroquilon
EPN	Oxamyl	Sethoxydim
Edifenphos	Phenmedipham	Thiabendazole
Ethion	Pirimicarb	Thiocyclam
Ethoprophos	Pyributicarb	Thiophanate-methyl
Fenamiphos	Thiobencarb	Triadimefon
Fenchlorphos	Thiobencarb sulfon	Tricyclazole
Fenitrothion	Thiram	Triflumizole
Fenitrothion oxon	6. Acid amides (n = 11)	Trifluralin
Fensulfothion	Alachlor	Triforine
Fenthion	Asulam	Vinclozolin
Glyphosate	Cafenstrole	
Iprobenfos	Flutolanil	
	Mefenacet	

EPN, *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate.

of the cloned hERβ cDNA was verified and was inserted into the mammalian expression vector pcDNA3.1Zeo(–) (Invitrogen, San Diego, CA, USA), creating pcDNAERβ.

The estrogen-responsive element (ERE)–containing reporter plasmid pGL3-tkERE and the androgen-responsive element (ARE)–containing reporter plasmid pIND-ARE were constructed as described previously (Kojima et al. 2003). pRL-SV40 containing the *Renilla* luciferase gene was purchased from Promega (Madison, WI, USA) and used as an internal control for transfection efficiency.

Reporter gene assays for hERα, hERβ, and hAR. The host CHO-K1 cells were plated in 96-well microtiter plates (Nalge Nunc, Rochester, NY, USA) at a density of 8,400 cells/well in phenol red–free DMEM/F-12 containing 5% CD–FBS (complete medium) 1 day before transfection. For detection of hERα or hERβ activity, cells were transfected with 5 ng pcDNAERα or 5 ng pcDNAERβ, 50 ng pGL3-tkERE, and 5 ng pRL-SV40 per well using the transfection reagent FuGene6 (Roche Diagnostics Corp., Indianapolis, IN, USA). For detection of hAR activity, cells were transfected with 2.5 ng pZeoSV2AR, 50 ng pIND-ARE, and 5 ng pRL-SV40 per well. After a 3-hr transfection period, cells were dosed with various concentrations of test compounds or with 0.1% DMSO (vehicle control) in complete medium. For measurement of the antagonistic activity to hERα, hERβ, and hAR, either 10^{-11} M E_2, 10^{-10} M E_2, or 10^{-10} M DHT was added to the cell cultures along with the test compound, respectively (Figure 1). After an incubation period of 24 hr, cells were rinsed with phosphate-buffered saline (pH 7.4) and lysed with passive lysis buffer (50 μL/well) provided with the Dual-Luciferase Reporter Assay kit (Promega). We measured the firefly luciferase activity with a MiniLumat LB 9506 luminometer (Berthold, Wildbad, Germany) before measuring *Renilla* luciferase activity in one reaction tube with 5-μL aliquots of cell lysates using the Dual-Luciferase Reporter Assay kit, following the manufacturer's instructions. The firefly luciferase activity was normalized based on the *Renilla* luciferase activity of the cotransfected pRL-SV40. The values shown are mean ± SD from at least three independent experiments.

We evaluated the results for the agonistic activities of the pesticides by relative activity, expressed as REC_{20} (20% relative effective concentration)—that is, the concentration of the test compound showing 20% of the activity of 10^{-10} M E_2, 10^{-9} M E_2, or 10^{-9} M DHT for ERα, ERβ, or AR, respectively. When the activity of the test compound was higher than REC_{20} within the concentration tested (~10^{-8} to 10^{-5} M), we judged the pesticide to be positive for activity. The results for the antagonistic activities of the pesticides were expressed as RIC_{20} (20% relative inhibitory concentration), that is, the concentration of the test compound showing 20% inhibition of the activity induced by 10^{-11} M E_2, 10^{-10} M E_2, or 10^{-10} M DHT for ERα, ERβ, or AR, respectively. When the activity of the test compound was higher than the RIC_{20} within the concentration tested, we judged the pesticide to be positive for inhibitory activity. To avoid cell toxicity by the pesticides, assays were performed for pesticides at concentrations ≤ 10^{-5} M.

Data analysis. We evaluated the statistical significance of differences using the Student's *t*-test (two-tailed, equal variance) calculated by software (Excel; Microsoft, Redmond, WA, USA). The level of significance was $p < 0.05$. Data are presented as the mean and, where shown, the SD of at least three separate experiments with duplicate wells.

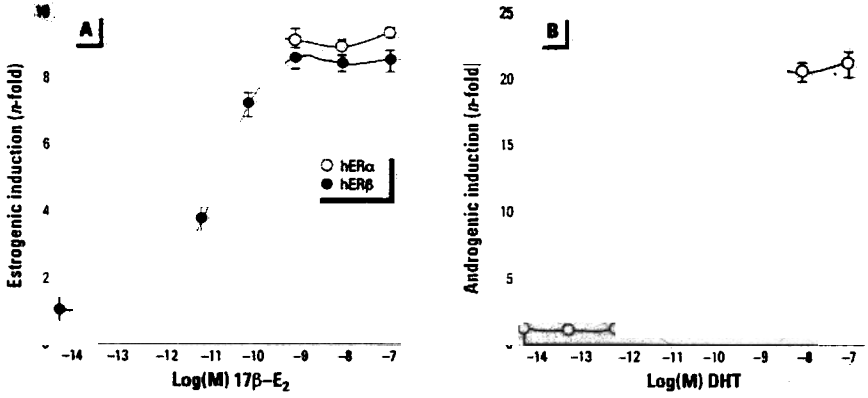

Figure 1. Dose–response curves of (*A*) 17β-E_2 and (*B*) 5α-DHT in ER and AR assays. CHO cells were transiently transfected with expression plasmids for (*A*) hERα or hERβ or (*B*) hAR plus relative receptor–responsive firefly luciferase reporter plasmids and a constitutively active *Renilla* luciferase expression plasmid (transfection and toxicity control). Cells were treated with increasing concentrations of 17β-E_2 or 5α-DHT to detect agonist activity. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Values represent the mean ± SD of three independent experiments and are presented as mean *n*-fold induction over the vehicle control.

Results

Response of 17β-E_2 in ERα and ERβ assays, and of 5α-DHT in AR assay. Figure 1A shows the dose-dependent transactivation of ERα and ERβ by 17β-E_2, indicating that both receptors can be activated at very low hormone concentrations. The maximal ERα activity was achieved at 10^{-10} M E_2 or more, exhibiting approximately 10-fold that of the control solvent. The maximal ERβ activity induced was 8.5-fold that of the solvent control at 10^{-9} M E_2 or more. Thus, E_2 was more potent for ERα than for ERβ. From these dose–response curves, REC_{20} values of E_2 for ERα and ERβ were deduced to be 2.5×10^{-12} M and 5.3×10^{-12} M, respectively.

Figure 1B shows the dose-dependent transactivation of AR by 5α-DHT. Its activity was detectable from 10^{-11} M DHT and reached a plateau at 10^{-9} M DHT. The maximum induction was 21-fold that of the control solvent. The REC_{20} value of DHT for AR was 3.1×10^{-11} M.

Estrogenic effects of the pesticides. Table 2 shows the REC_{20} values and relative estrogenic activities at 10^{-5} M of pesticides evaluated as positive for ERα agonistic activity. As shown in Table 2, 47 of the 200 pesticides were found to induce estrogenic activity in the ERα assay. A comparison of the potency of estrogenic activities among these active pesticides shows that the REC_{20} values of *o,p'*-DDT, β-BHC, methoxychlor, and α-endosulfan among the organochlorine pesticides, the CNP metabolite CNP-amino among the diphenyl ether pesticides, and butamifos among the organophosphorus pesticides were all lower than 10^{-6} M, indicating that they possess potent estrogenic activity.

The results of ERβ agonistic activity are presented in Table 3. Thirty-three of 200 pesticides increased the ERβ-mediated transactivation gene response. Twenty-nine of these pesticides also have estrogenic activity via ERα (Table 2). The REC_{20} values of β-BHC and *o,p'*-DDT among the organochlorine pesticides, CNP-amino among the diphenyl ether pesticides, and methiocarb among the carbamate pesticides were lower than 10^{-6} M. However, butamifos, which showed potent ERα agonistic activity among the organophosphorus pesticides, was inactive in the ERβ assay. Dose–response curves of β-BHC, δ-BHC, and methiocarb for ERα and ERβ are shown in Figure 2. These pesticides stimulated ERβ more strongly than they did ERα.

Antiestrogenic effects of the pesticides. Of 200 test pesticides, five (cyhalothrin, deltamethrin, alachlor, pyrazoxyfen, and triflumizole) showed antiestrogenic properties in the hERα transactivation assay with 10^{-10} M E_2. As shown in Figure 3A, 10^{-5} M of these pesticides significantly inhibited the estrogenic response by 10^{-11} M E_2, as did the well-known

ER antagonist tamoxifen (10^{-8} and 10^{-7} M). The RIC_{20} values of cyhalothrin, deltamethrin, alachlor, pyrazoxyfen, triflumizole, and tamoxifen for hERα were 9.0×10^{-6} M, 8.1×10^{-6} M, 4.5×10^{-6} M, 6.0×10^{-6} M, 9.8×10^{-6} M, and 3.2×10^{-9} M, respectively.

In the hERβ transactivation assay of the 200 tested pesticides, only methoxychlor and pyrazoxyfen were antiestrogenic. As shown in Figure 3B, 10^{-5} M methoxychlor or pyrazoxyfen inhibited by more than 20% the estrogenic activity induced by 10^{-10} M E_2. In this assay, tamoxifen also showed antiestrogenic activities at concentrations of 10^{-8} and 10^{-7} M. The RIC_{20} values of methoxychlor, pyrazoxyfen, and tamoxifen for hERβ were 9.0×10^{-6} M, 7.8×10^{-6} M, and 6.0×10^{-9} M, respectively.

Androgenic effects of the pesticides. None of the pesticides tested showed androgenic transcriptional activity of more than 20% that induced by 10^{-9} M DHT at the tested concentrations (data not shown).

Antiandrogenic effects of the pesticides. We tested 200 pesticides for their inhibitory effect on the androgenic activity induced by DHT (10^{-10} M). The RIC_{20} values and relative luciferase activities (RLA) of 66 pesticides evaluated as having an inhibitory effect are summarized in Table 4. In particular, 13 pesticides (*o,p'*-DDT, *p,p'*-DDE, *p,p'*-DDT, chloropropylate, CNP, chlomethoxyfen, nitrofen, CNP-amino, oxyfluorfen, fenitrothion, vinclozolin, procymidone, and bromopropylate) showed a potent antiandrogenic effect with $RIC_{20} < 10^{-6}$ M. Among these active pesticides, the RIC_{20} of two diphenyl ether-type herbicides, CNP and chlomethoxyfen, were 4.3×10^{-8} M and 6.8×10^{-8} M, respectively, which is distinctly more potent than known AR antagonists such as vinclozolin and *p,p'*-DDE (1.6×10^{-7} M and 6.5×10^{-7} M, respectively). In addition, weak antiandrogen effects with $RIC_{20} > 10^{-6}$ M were found in 53 pesticides: 10 organochlorines, 2 diphenyl ethers, 18 organophosphorus pesticides, 4 pyrethroids, 2 carbamates, 3 acid amides, 5 ureas, and 9 others.

Both ER agonists and AR antagonists in the pesticides. Table 5 summarizes the 34 pesticides exhibiting dual activities as ER agonists and AR antagonists. Among these pesticides, organochlorine and organophosphorus pesticides were predominant. Above all, *o,p'*-DDT and CNP-amino were the most potent pesticides, having both estrogenic and antiandrogenic activities.

Cytotoxicity was not observed for any of the tested compounds at the selected dose range (data not shown).

Discussion

To our knowledge, there are two reports on the screening of the endocrine-disrupting effects of a large number of chemicals: on estrogenic activity of 514 chemicals using a yeast two-hybrid assay (Nishihara et al. 2000) and on binding ability of 188 chemicals to rat ER using a competitive binding assay (Blair et al. 2000). However, in these studies 118 pesticides of 514 chemicals and 20 pesticides of 188 chemicals showed little estrogenic activity or little binding ability to ER, respectively. In addition, there is no report on screening of ERβ-mediated estrogenic activity from a large number of chemicals. We previously developed highly sensitive and specific reporter gene assays for ERα and AR (Kojima et al. 2003), and in the present study we established the ERβ assay by constructing the hERβ expression plasmid pcDNAERβ in our screening of 200 pesticides for their estrogenicity via hERα/β and androgenicity via hAR. As a result, we found estrogenic activity for hERα in 47 pesticides and for hERβ in 33 pesticides, and antiestrogenic activity for hERα and hERβ in five and two pesticides, respectively. In the AR assay, although none of the tested pesticides showed AR agonistic activity, 66 of the 200 test pesticides surprisingly showed antiandrogenic activities. Thus, a number of pesticides were newly found to possess ER agonistic and/or AR antagonistic activities in addition to the pesticides already reported to be estrogenic and antiandrogenic. This suggests that our reporter gene assays are highly sensitive and specific.

We classified 200 pesticides into nine groups according to their chemical structure:

Table 2. Responses induced by pesticide testing positive in the ERα transactivation assay.

Group[a], compound	REC_{20}[b] (M)	RLA[c] (%)
	2.5×10^{-12}	
	4.5×10^{-8}	
	3.5×10^{-7}	
	5.6×10^{-7}	
	7.4×10^{-7}	
	1.1×10^{-6}	
	1.2×10^{-6}	
	1.3×10^{-6}	
	1.8×10^{-6}	
	1.8×10^{-6}	
	2.0×10^{-6}	
	2.1×10^{-6}	
	2.2×10^{-6}	
	3.2×10^{-6}	
	4.0×10^{-6}	
	6.0×10^{-6}	
	7.1×10^{-6}	
	7.8×10^{-6}	
	8.4×10^{-6}	
	3.7×10^{-7}	
	4.2×10^{-6}	
	7.8×10^{-6}	
	8.7×10^{-7}	
	1.3×10^{-6}	
	1.3×10^{-6}	
	2.1×10^{-6}	
	2.2×10^{-6}	
	2.5×10^{-6}	
	2.7×10^{-6}	
	2.7×10^{-6}	
	3.7×10^{-6}	
	4.1×10^{-6}	
	4.2×10^{-6}	
	4.8×10^{-6}	
	5.7×10^{-6}	
	6.2×10^{-6}	
	6.6×10^{-6}	
	7.5×10^{-6}	
	3.7×10^{-6}	
	5.7×10^{-6}	
	5.9×10^{-6}	
	8.1×10^{-6}	
	8.4×10^{-6}	
	7.2×10^{-6}	
	1.6×10^{-6}	
	1.7×10^{-6}	
	2.5×10^{-6}	
	3.1×10^{-6}	

Abbreviations: EPN, *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate; RLA, relative luciferase activity.
[a]Nine compound groups are listed in Table 1. [b]Concentration of the test compound showing 20% of the agonistic activity of 10^{-10} M E_2. [c]Percentage response at a concentration of 10^{-5} M with 100% activity defined as the activity achieved with 10^{-10} M E_2. [d]RLA of E_2 is represented as the activity at a concentration of 10^{-10} M.

Table 3. Responses induced by pesticide testing positive in the ERβ transactivation assay.

Group[a], compound	REC_{20}[b] (M)	RLA[c] (%)

Abbreviations: EPN, *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate; RLA, relative luciferase activity.
[a]Nine compound groups are listed in Table 1. [b]Concentration of the test compound showing 20% of the agonistic activity of 10^{-9} M E_2. [c]Percentage response at a concentration of 10^{-5} M with 100% activity defined as the activity achieved with 10^{-9} M E_2. [d]RLA of E_2 is represented as the activity at a concentration of 10^{-9} M.

organochlorines, diphenyl ethers, organophosphorus pesticides, pyrethroids, carbamates, acid amides, triazines, ureas, and others. Organochlorine-type pesticides should be of the most concern among the nine groups of pesticides suggested as candidates to be endocrine disruptors, because of their global distribution by widespread use and bioaccumulation through the ecosystem by high lipophilic property (Kutz et al. 1991; Simonich and Hites 1995). Several of these compounds (DDT, methoxychlor, BHC, endosulfan, and dieldrin) have been reported to possess estrogenic activity by studies with animals and cells (Bulger et al. 1978; Coosen and Velsen 1989; Soto et al. 1995; Welch et al. 1969). We have also demonstrated that *o,p'*-DDT, β-BHC, methoxychlor, α-endosulfan, and *cis*(*trans*)-chlordane exert transcriptional reporter activity via ERα and/or ERβ by our assays. In addition, we newly identified estrogenic organochlorine pesticides such as dicofol, chloropropylate, and chlorobenzilate, whose chemical structures resemble those of DDT and its isomer (Figure 4). Among BHC isomers, β-BHC is most prevalent in the fatty tissues because of its greater stability, lipophilicity, and accumulation potential (Dejonckheere et al. 1978). In the present study, β-BHC and δ-BHC exerted potent estrogenic activity especially via ERβ (Figure 2) but showed no effect in the androgen assay. Moreover, we also found 14 antiandrogenic pesticides among the organochlorine pesticides. The antiandrogenic properties of DDT isomers, methoxychlor, dieldrin, and endosulfan have already been reported (Andersen et al. 2002; Kelce et al. 1995; Maness et al. 1998), but in the present study several other pesticides were newly defined as AR antagonists. Although many organochlorine pesticides have weak hormonal activity, their lipophilic nature and long half-lives allow them to accumulate in the fatty tissues of the body, increasing their concentration and bioavailability.

Diphenyl ether pesticides are no longer used, but a few decades ago this type of chemical was extensively used as an herbicide due to its low cost and toxicity. We have reported that a diphenyl ether–type pesticide, CNP, and its amino derivative (CNP-amino) act as both ERα agonists and AR antagonists *in vitro* (Kojima et al. 2003). In the present study, we additionally found that CNP-amino, but not CNP, possessed an ERβ agonistic effect, and that except for fluazifop-butyl, which demonstrated the weak ERα agonistic activity, no other diphenyl ether–type pesticides showed estrogenic activity. This suggests that CNP-amino itself, rather than the structure of diphenyl ether, may contribute to the transactivation of ERs. Nevertheless, the greatest concern of diphenyl ether pesticides as endocrine-disrupting agents should reside in their potent AR antagonist activity. Recently, Tomura et al. (2001) demonstrated that a diphenyl ether herbicide, nitrofen, was antiandrogenic by transactivation assay and three-dimensional image analysis using COS-7 simian renal carcinoma cells. In the present study, seven of 11 diphenyl ether pesticides, including CNP, CNP-amino, and nitrofen showed antiandrogenic activity, and in

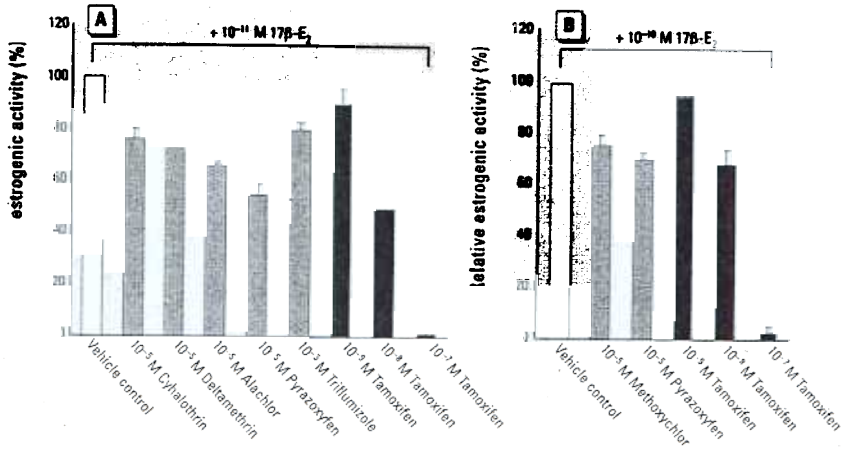

Figure 3. Antagonistic effects of pesticides in the hERα and hERβ transactivation assays. (*A*) CHO cells, transiently cotransfected with pcDNAERα, pGL3-tkERE, and pRL-SV40, were incubated with the vehicle control (0.1% DMSO) or 10^{-5} M of cyhalothrin, deltamethrin, alachlor, pyrazoxyfen, or triflumizole in the presence of 10^{-11} M E_2. Effect of ER antagonist tamoxifen (~10^{-9} to 10^{-7} M) was also measured as a positive control. Values represent the mean ± SD of three independent experiments and are presented as percent induction, with 100% activity defined as the activity achieved with 10^{-11} M E_2. (*B*) CHO cells, transiently cotransfected with pcDNAERβ, pGL3-tkERE, and pRL-SV40, were incubated with the vehicle control (0.1% DMSO) or 10^{-5} M of methoxychlor or pyrazoxyfen in the presence of 10^{-10} M E_2. Effect of ER antagonist tamoxifen (~10^{-9} to 10^{-7} M) was also measured as a positive control. Values represent the mean ± SD of three independent experiments and are presented as percent induction, with 100% activity defined as the activity achieved with 10^{-10} M E_2.
*Significantly different ($p < 0.05$) from vehicle control (=100%).

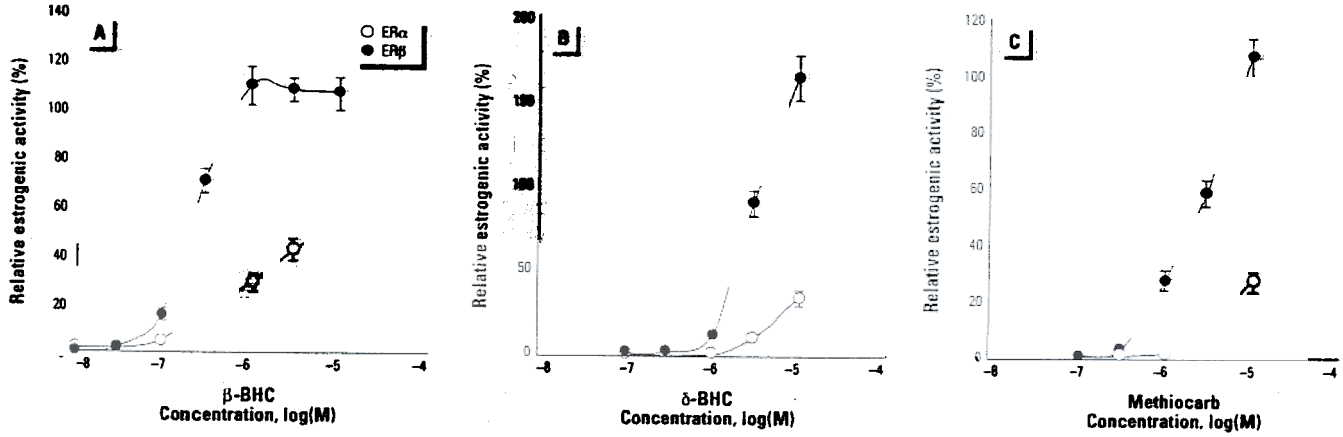

Figure 2. Dose–response curves of (*A*) β-BHC, (*B*) δ-BHC, and (*C*) methiocarb on hERα- and hERβ-mediated estrogenic activities. CHO cells were transiently transfected with pcDNAERα or pcDNAERβ, pGL3-tkERE, and pRL-SV40 as described in "Materials and Methods." Cells were incubated with various concentrations of (*A*) β-BHC, (*B*) δ-BHC, or (*C*) methiocarb. Values represent the mean ± SD of three independent experiments and are presented as percent induction, with 100% activity defined as the activity achieved with 10^{-10} M and 10^{-9} M E_2 for ERα and ERβ, respectively.

particular, the antiandrogenic activities of CNP and chlomethoxyfen were more potent than those of known AR antagonists such as vinclozolin and *p,p'*-DDE. Chlomethoxyfen is structurally similar to CNP and nitrofen as well as the antiandrogen drug flutamide and the organophosphorus insecticide fenitrothion, all of which commonly contain nitrobenzene in the molecular structure (Figure 4). This may be the key point in screening AR antagonists from the multitude of chemicals (Kojima et al. 2003).

Organophosphorus pesticides are widely used in both agriculture and pest control. We found that a number of organophosphorus-type pesticides possess estrogenic and/or antiandrogenic activities. To date, it has been reported that tolclofos-methyl and quinalphos act as ER agonists (Andersen et al 2002; Chatterjee et al. 1992) and that fenitrothion, parathion, and methyl parathion act as AR antagonists (Sohoni et al. 2001; Tamura et al 2001). We also found that tolclofos-methyl and quinalphos have estrogenic activities via ERα and ERβ, and that fenitrothion, parathion, and methyl parathion have antiandrogenic activities via AR. Moreover, here we provide new evidence that butamifos, prothiofos, leptophos, cyanofenphos, ethion, bromophosethyl, *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate (EPN), and dichlofenthion induce ERα-mediated transcriptional activity at concentrations lower than that of quinalphos. In addition, we found that 19 organophosphorus pesticides, including fenitrothion, parathion, and methyl parathion, possess antiandrogenic activity. Thus, the similarity in chemical structure among these pesticides may be the primary cause for their estrogenicity and/or antiandrogenicity. Interestingly, organophosphorus pesticides displaying these effects commonly contain a thiophosphoryl residue (P = S), as shown in Figure 4, whereas pesticides having an oxophosphoryl residue (P = O) such as prothiofos oxon, tolclofos-methyl oxon, acephate, and propaphos show few effects. This indicates that the estrogenic and/or antiandrogenic activities of parent compounds may disappear through oxidizing metabolism in the environment or body.

Pyrethroid and organophosphorus pesticides are the pesticides used most in Japan and other countries. Several pyrethroid pesticides (fenvalerate, permethrin, and cypermethrin) have been reported to show estrogenic activities in MCF-7 cell proliferation and transactivation assays (Chen et al. 2002; Garey and Wolff 1998; Go et al. 1999). In contrast, Saito et al. (2000) reported that fenvalerate did not have estrogenic activity *in vitro* at a concentration of 10^{-5} M. In our assays, five pyrethroid pesticides including fenvalerate were shown to increase transcriptional activity via ERα, and two pyrethroid pesticides decreased ERα activity induced by 10^{-11} M E_2. This suggests that several pyrethroid pesticides may act as weak ER agonists or antagonists. In addition, four pyrethroid pesticides were newly found to have weak antiandrogenic activity, and three of them (fenvalerate, flucythrinate, and cyfluthrin; Figure 4) displayed pleiotropic effects via both ERα and AR.

Klotz et al. (1997) reported that several carbamate insecticides (e.g., carbaryl, methomyl,

Table 4. Inhibitory effects of 66 pesticides on AR transcriptional activity induced by DHT.

Group[a], compound	RIC_{20}[b] (M)	RLA[c] (%)	RIC_{20}[b] (M)	RLA[c] (%)
DHT alone			4.9×10^{-8}	
1. *o,p'*-DDT	5.5×10^{-7}		5.5×10^{-6}	
p,p'-DDE	6.5×10^{-7}		5.7×10^{-6}	
p,p'-DDT	7.1×10^{-7}		7.4×10^{-6}	
Chloropropylate	7.2×10^{-7}		7.8×10^{-6}	
Chlorobenzilate	1.2×10^{-6}		8.7×10^{-6}	
Heptachlor epoxide	1.3×10^{-6}		9.4×10^{-6}	
Dicofol	1.6×10^{-6}		6.6×10^{-6}	
p,p'-DDD	1.8×10^{-6}		6.9×10^{-6}	
β-Endosulfan	2.0×10^{-6}		8.4×10^{-6}	
Methoxychlor	2.1×10^{-6}		9.2×10^{-6}	
trans-Chlordane	2.4×10^{-6}		2.8×10^{-6}	
cis-Chlordane	2.5×10^{-6}		9.4×10^{-6}	
Dieldrin	2.8×10^{-6}		5.4×10^{-6}	
α-Endosulfan	6.9×10^{-6}		5.6×10^{-6}	
2. Chlornitrofen	4.3×10^{-8}		9.6×10^{-6}	
Chlomethoxyfen	6.8×10^{-8}		1.4×10^{-6}	
Nitrofen	3.4×10^{-7}		1.5×10^{-6}	
CNP-amino	8.3×10^{-7}		2.0×10^{-6}	
Oxyfluorfen	8.7×10^{-7}		3.4×10^{-6}	
Bifenox	3.2×10^{-6}		8.7×10^{-6}	
Acifluorfen-methyl	8.9×10^{-6}		1.6×10^{-7}	
3. Fenitrothion	1.8×10^{-7}		2.0×10^{-7}	
Anilofos	1.9×10^{-6}		5.3×10^{-7}	
EPN	1.9×10^{-6}		1.2×10^{-6}	
Prothiofos	2.2×10^{-6}		2.6×10^{-6}	
Parathion	2.2×10^{-6}		3.5×10^{-6}	
Methyl parathion	2.3×10^{-6}		4.2×10^{-6}	
Tolclofos-methyl	2.8×10^{-6}		4.9×10^{-6}	
Piperophos	3.0×10^{-6}		5.0×10^{-6}	
Ethion	3.3×10^{-6}		6.2×10^{-6}	
Butamifos	3.3×10^{-6}		7.0×10^{-6}	
Phosalone	4.5×10^{-6}		7.8×10^{-6}	
Dichlofenthion	4.8×10^{-6}			

Abbreviations: EPN, *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate; RLA, relative luciferase activity.
[a]Nine compound groups are listed in Table 1. [b]Concentration of the test compound showing 20% inhibition of the androgenic activity induced by 10^{-10} M DHT. [c]Percentage response at a concentration of 10^{-5} M with 100% activity defined as the activity achieved with 10^{-10} M DHT.

Table 5. Thirty-four pesticides possessing both estrogenic and antiandrogenic activities *in vitro*.

Pesticide	hERα	hERβ	hAR
Group 1			
cis-Chlordane	↑	↑	
trans-Chlordane	↑	↑	
o,p'-DDT	↑↑		↓↓
p,p'-DDT	↑	↑	↓↓
p,p'-DDE	↑	↑	↓↓
p,p'-DDD	↑	↑	↓
Chlorobenzilate	↑		↓
Chloropropylate	↑	↑	↓↓
Dicofol	↑	↑	
Dieldrin	↑		
α-Endosulfan	↑↑		
β-Endosulfan			
Heptachlor epoxide			
Methoxychlor	↑		
Group 2			
Chlornitrofen (CNP)	↑		↓↓
CNP-amino		↑↑	↓↓
Group 3			
Bromophos-ethyl			
Butamifos	↑↑		↓
Dichlofenthion	↑		↓
EPN	↑		↓
Ethion	↑		↓
Isofenphos			
Leptophos	↑		↓
Prothiofos	↑		↓
Quinalphos	↑		
Tolclofos-methyl	↑		
Group 4			
Fenvalerate			
Cyfluthrin			
Flucythrinate			
Group 5			
Methiocarb		↑↑	
Group 6			
Thenylchlor	↑		
Group 9			
Bromopropylate	↑		
Fenarimol	↑		
Pendimethalin	↑		

Symbols: ↑↑, agonistic effect ($REC_{20} \leq 10^{-6}$ M); ↑, agonistic effect (10^{-6} M < $REC_{20} \leq 10^{-5}$ M); ↓↓, antagonistic effect ($RIC_{20} \leq 10^{-6}$ M); ↓, antagonistic effect (10^{-6} M < $RIC_{20} \leq 10^{-5}$ M); —, no effect. EPN, *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate.

oxamyl) decreased estrogen- or progesterone-responsive reporter genes at concentrations of 10^{-7} M in breast (MCF-7) and endometrial (Ishikawa) cancer cells. However, Andersen et al. (2002) reported that methomyl induced no significant effects in proliferation and ER transactivation assays using MCF-7 cells and that methiocarb showed both estrogenic and weak antiandrogenic properties. In our study, of the 22 carbamates tested only methiocarb (see Figure 4) showed both estrogenic and antiandrogenic activities, and other carbamate pesticides showed no ER or AR activity. Thus, our results support the evidence of Andersen et al. (2002) but not that of Klotz et al. (1997).

With regard to acid amide–type pesticides, only one relevant study was available. Vonier et al. (1996) reported the interaction of the herbicide alachlor with the estrogen and progesterone receptors from the oviduct of the American alligator. They showed that this pesticide competed with E_2 for binding to the ER, but the binding affinity was about 3,500-fold lower than that of E_2. In our assays using hERs and hAR, alachlor showed both antiestrogenic activity via ERα and antiandrogenic activity. This suggests that alachlor can interact not only with alligator ER but also with hERα and hAR. Furthermore, among 13 acid amide pesticides, we newly found thenylchlor to have both estrogenic and antiandrogenic activities, and mefenacet to possess antiandrogenic activity.

Urea-type pesticides are mainly used as herbicides. Bauer et al. (1998) reported evidence that propanil (DCPA), linuron, and diuron in phenyl urea herbicide have the ability to bind to AR. In addition, recent reports have shown linuron and prochloraz to be antiandrogenic *in vitro* and *in vivo* (Lambright et al. 2000; Vinggaard et al. 2002). In our AR assay, five urea-type herbicides (DCPA, pencycuron, linuron, prochloraz, and diuron) inhibited transcriptional activity by DHT, and the antiandrogenic activities of DCPA and pencycuron were more potent than those of linuron and prochloraz, which have been shown to be antiandrogenic *in vivo*. On chemical structure, these pesticides have some similarities (Figure 4). These suggest that DCPA and pencycuron would also show antiandrogenic activity *in vivo* and should therefore be considered endocrine disruptors.

Among the triazine-type pesticides, atrazine, which is the most widely used herbicide in the United States, has been reported to be antiestrogenic by yeast transactivation assay (Tran et al. 1996). In the present study, seven triazine-type pesticides were tested, but none showed any ER or AR activity. Friedmann (2002) recently reported that atrazine acts as an endocrine disruptor in rat males by directly inhibiting Leydig cell testosterone production. Hayes et al. (2002) hypothesize that atrazine induces aromatase and promotes the conversion of testosterone to estrogen in *Xenopus laevis*. Thus, this type of pesticide may exert hormonal activity through mechanisms other than those associated with ER and AR.

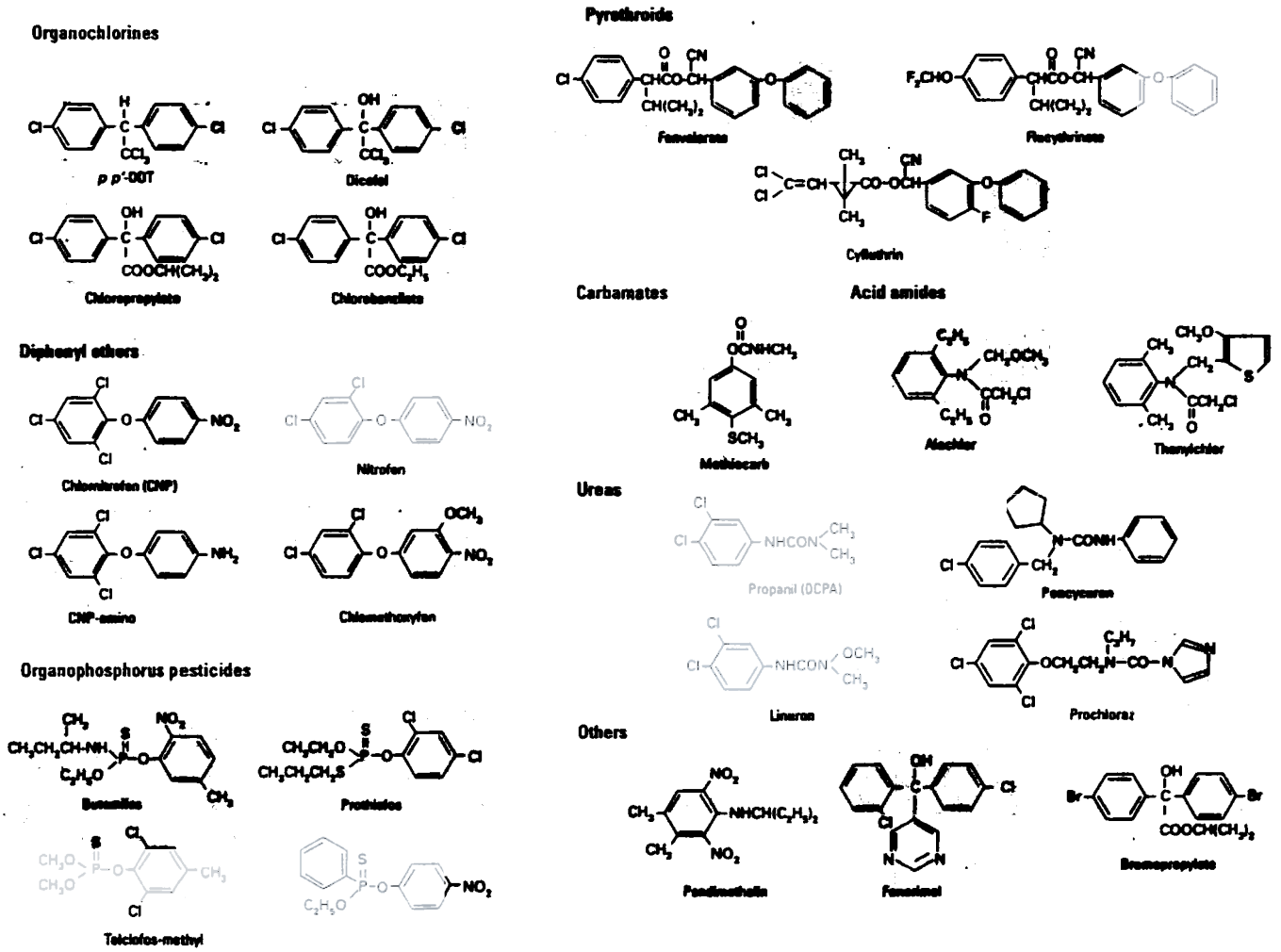

Figure 4. Chemical structures of some exemplar pesticides showing estrogenic and/or antiandrogenic activity.

In the present study, 44 pesticides that could not be classified into the above eight groups were collectively grouped as "others." The dicarboxyimide fungicides vinclozolin and procymidone are structurally similar and showed potent antiandrogen effects. This result corresponded to those of other studies (Kelce et al. 1994; Ostby et al. 1999). In addition, bromopropylate, fenarimol, and pendimethalin showed both estrogenic and antiandrogenic properties (Figure 4). Because the chemical structures of bromopropylate and chloropropylate are similar to those of DDT isomers, it was thought that the two pesticides would have similar effects. The *in vitro* estrogenic and antiandrogenic effects of the fungicide fenarimol have been also described by Andersen et al. (2002). The effects of fenarimol in our assays were more potent than described in that study, likely because of the difference in sensitivity of the assay systems. The herbicide pendimethalin has not been reported as having endocrine-disrupting effects, and thus we are the first to demonstrate the effects of this pesticide.

To date, no AR agonists have been found among environmental chemicals, and in this study we also failed to isolate an AR agonist from among 200 pesticides tested but identified 66 antiandrogenic pesticides. In addition, although there are many ER agonistic pesticides, there are also quite a few pesticides with antiestrogenic properties. This phenomenon is, as Sohoni and Sumpter (1998) pointed out, quite enigmatic. Furthermore, we demonstrated that a lot of pesticides possessed both estrogenic and antiandrogenic activities. Taken together, most of these chemical compounds may act as ER agonists and/or AR antagonists in the environment, a situation leading to feminization in animals.

Our experiments demonstrate that many pesticides possess *in vitro* estrogenic and antiandrogenic activities through ERs and/or AR. Although it appears that various pesticides exert hormonal effects at concentrations orders of magnitude higher than that required for physiologic hormones, wide exposure to large numbers of these pesticides may have additive and synergistic effects.

The first aim of this study was the comprehensive evaluation of 200 pesticides for *in vitro* estrogenicity and androgenicity under the same conditions using one highly sensitive and specific assay method. If different cells and plasmids were used in the assay, different results may be produced. However, we believe that the reporter gene assays in the present study are useful for identifying endocrine disruptors via ERs and AR from a large number of chemicals. Such hormonal effects are expected to be found not only in pesticides but also in other chemicals in the environments. The second aim was the search for a relationship between chemical structure and hormonal activity. In fact, we found it in a number of pesticides. This is an important point in identifying endocrine disruptors from the multitude of chemicals commonly in use. We herein propose that many compounds should be tested using the same method and under the same conditions to prevent confusion resulting from the use of different methods, and an international agreement should be reached for this purpose.

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Environmental and Molecular Mutagenesis 32:39–46 (1998)

Cytogenetic Damage and Induction of Pro-Oxidant State in Human Lymphocytes Exposed In Vitro to Gliphosate, Vinclozolin, Atrazine, and DPX-E9636

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We analyzed chromosome aberrations (CAs), sister chromatid exchanges (SCEs), mitotic index (MI), and glucose 6-phosphate dehydrogenase (G6PD) enzyme activity in human peripheral lymphocytes from three healthy donors exposed in vitro to different concentrations of gliphosate, vinclozolin, atrazine, and DPX-E9636. The pesticides gliphosate, vinclozolin, and atrazine have been studied in a broad range of genetic tests with predominantly conflicting or negative results, whereas little is known about the genotoxicity of DPX-E9636. In our experimental conditions, each chemical compound tested produced a dose-related increase in the percent of aberrant cells and an increase of SCE/cell. Furthermore, at the highest concentrations of vinclozolin, atrazine, and DPX-E9636, we observed a significant reduction of the mitotic index. The increase of G6PD activity in exposed lymphocyte cultures strongly indicated an induction of a pro-oxidant state of the cells as an initial response to pesticide exposure. Environ. Mol. Mutagen. 32:39–46, 1998

Key words: genotoxicity; chromosome aberrations; sister chromatid exchanges; glucose 6-phosphate dehydrogenase; oxidative stress; pesticides

INTRODUCTION

Extensive studies have been carried out to evaluate the mutagenic potential of the pesticides gliphosate, vinclozolin, and atrazine in vitro and in vivo. Most of the reports have indicated that gliphosate shows minimal genotoxic activity [Vigfusson and Vyse, 1980; Li and Long, 1988; Rank et al., 1993], whereas controversial results have been obtained on vinclozolin [Chiesara et al., 1982; Perocco et al., 1993; Hrelia et al., 1996] and atrazine [Adler, 1980; Meisner et al., 1992; Brusick, 1994] in different experimental systems ranging from bacterial to mammalian assays. On the other hand, no data are available on the mutagenic potential of the recently introduced DPX-E9636 in mammals.

The above considerations prompted us to improve our knowledge of the genotoxicity of the four pesticides and to gain insight into their possible mechanism of action. Thus, we investigated the genotoxic potential of increasing concentrations of gliphosate, vinclozolin, atrazine, and DPX-E9636 in in vitro cultures of human lymphocytes by using as genetic endpoints chromosome aberration (CA) and sister chromatid exchange (SCE) frequencies and, as an indicator of the change in the cell redox state, glucose 6-phosphate dehydrogenase (G6PD) enzyme activity. G6PD catalyzes the first and rate-limiting step of the hexose monophosphate (HMP) shunt and produces reducing power, in the form of NADPH, which is used by cells to drive the enzymatic reactions required to remove reactive oxygen intermediates (ROIs) [Meister and Anderson, 1983] and is necessary to maintain the intracellular pool of reduced glutathione (GSH), which is the main antioxidant molecule present in the cell cytoplasm [Meister, 1988]. Recent reports have clearly demonstrated that the cell pro-oxidant state and, thus, GSH depletion, is always followed by increased G6PD activity, indicating that G6PD is functioning as an antioxidant enzyme [Clancy et al., 1994; Banki et al., 1996; Ursini et al., 1997]. Furthermore, it has been observed that eukaryotic cells, bearing a genetically determined G6PD null mutation, are extremely sensitive to oxidative stress [Pandolfi et al., 1995]. We therefore examined the cytogenetic

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TABLE Distinctive Features of Pesticides Assayed

Common name	IUPAC name	Chemical class	CAS registry number	Activity
Gliphosate	N-(phosphonomethyl)glycine	Phosphorganics	1071-83-6	Herbicide
Vinclozolin	3-(3,5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-2,4-dione	Nitrogen-organic-heterocycles	50471-44-8	Fungicide
Atrazine	2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine	Nitrogen-organic-heterocycles	1912-24-9	Herbicide
DPX-E9636	1-(4,6-dimethoxypyrimidin-2-yl)-3-(ethylsulfonyl-2-pyridysulfonyl)urea	Sulfonylureas	122931-48-0	Herbicide

effects and measured G6PD activity in human lymphocytes after pesticide treatment. Our data strongly indicate either a genotoxic effect of all pesticides or a change in the cell redox state.

MATERIALS AND METHODS

Chemicals

Gliphosate, vinclozolin, and atrazine utilized in this study were purchased from Lab Service Analytica (Bologna, Italy) and DPX-E9636 from Du Pont (Paris, France) (purity of all active agents ≥ 98%). IUPAC names and chemical classes are given in Table I. Atrazine and vinclozolin were dissolved in DMSO (Fluka, Buchs, Switzerland) 0.3% final concentration in culture, whereas DPX-E9636 and gliphosate were dissolved in ethanol (Sigma, St. Louis, MO, USA) (0.1% final concentration) and in sterile water, respectively. For CA and SCE analysis, pesticides were added immediately after phytohemagglutinin stimulation and left throughout the culture period.

Lymphocyte Cultures

Lymphocytes from three healthy donors were separated by Ficoll-Hypaque gradient density following standard techniques [Böyum, 1968]. Briefly, 1 ml of buffy coat was cultured in 9 ml of RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum (FCS), 10 μg/ml L-glutamine, and 10 μg/ml phytohemagglutinin (PHA, M form), all obtained from Gibco (New York, NY, USA). Cells were cultured in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C. To eliminate variable sources due to culture conditions, exposed and control cultures were concurrent; the same batches of culture medium and an equal number (2×10^6) of cells/plate were used.

CA, SCE, and MI Analysis

Cells were cultured for 72 hr at 37°C for CA studies. After 70 hr of incubation, colcemid (Gibco, 50 μl for 10 ml cultures) was added. Lymphocytes were collected by centrifugation, resuspended in prewarmed hypotonic solution (0.075 M KCl) for 20 min, fixed in methanol/acetic acid (3:1) for 10 min, and stained with a 5% Giemsa solution (pH 6.8) for 10 min.

For SCE analysis, 30 hr to prior harvesting BrdU (Sigma) was added to each culture (10 μg/ml final concentration). The cultures were protected from light to avoid photolysis. Colcemid was added during the final 2 hr (50 μl for 10 ml cultures); harvested cells were treated with hypotonic solution and fixed with methanol/acetic acid (3:1). Air-dried slides were stained with a 0.2% acridine orange solution in phosphate buffer (pH 6.8) and sealed with paraffin.

From the four selected concentrations and from each subject, 50 well-spread metaphases with 46 chromosomes were scored on blindly coded slides for CAs (chromosome breaks, chromatid breaks, and fragments) and for SCEs. Gaps were recorded but not included either in the calculation of aberration frequency or in the percentage of aberrant cells [Preston et al., 1987].

The MI was evaluated by counting at least 1,000 cells per treatment: the number of metaphases was divided by the total number of cells counted to give percent mitosis.

G6PD Enzyme Assay

PHA stimulated cells were cultured for 48 hr at 37°C. An equal number of lymphocytes were plated and, except for the controls, treated with increasing concentrations of each pesticide. After 6 hr treatment, the cells were collected and centrifuged. G6PD activity was determined, as described previously [Battistuzzi et al., 1985], by measuring the production rate of NADPH. Since 6-phosphogluconate dehydrogenase (6PGD), the second enzyme of the pentose phosphate pathway, also produces NADPH, both 6PGD and total dehydrogenase activity (G6PD plus 6PGD) were determined separately, as previously reported, in order to obtain accurate enzyme activity for G6PD [Stanton et al., 1991; Tian et al., 1994].

Treatment with the antioxidant N-acetyl-cysteine (NAC) (Sigma) was performed by adding 30 mM final concentration dissolved in PBS (Gibco) 1 hr prior to pesticide exposure and left during the incubation period [Schreck et al., 1991].

Cell survival was determined by the Trypan-blue (0.5% solution, Serva, Heidelberg, Germany) exclusion technique.

Statistical Analysis

The comparison between exposed and control groups for each pesticide and for each dose was performed according to the two-tailed paired Student's *t*-test.

RESULTS

Cytogenetic Analysis

In order to assess the genotoxic activity of the chemicals on human lymphocyte cultures, we first carried out a preliminary study to determine the higher concentrations at which we were still able to observe a sufficient number of metaphases either for CAs or for SCEs. The concentration range 5–51 μM and 72-hr culture time were chosen for best demonstrating cytogenetic damage.

Table II shows the results of the induction of chromosome aberrations and the mitotic index in in vitro lympho-

TABLE II. Chromosomal Aberrations in Controls and 72-hr Lymphocyte Cultures Exposed to Vinclozolin, Gliphosate, and Atrazine

Treatment	Concentration (μM)	No. of subjects	% of aberrant cells[a] (Mean ± SD)	Chromosomal aberrations: Breaks: Chromosome type	Breaks: Chromatid type	Fragments	Gaps	No. of total aberrations	Aberration frequency[b] (Mean ± SD)	Mitotic index[c] (Mean ± SD)
Control	—	3	0.7 ± 1.2	—	1	—	3	1	0.7 ± 1.2	75.1 ± 6.7
DMSO	(0.3%)	3	0.7 ± 1.2	—	2	—	3	2	1.3 ± 2.3	65.7 ± 3.3
Gliphosate	5.0	3	3.3 ± 4.2	2	3	—	2	5	3.3 ± 4.2	64.0 ± 2.8
	8.5	3	6.7 ± 3.1*	1	12	1	8	14	9.3 ± 3.1*	60.5 ± 3.5
	17.0	3	23.3 ± 3.1*	25	20	—	—	45	30.0 ± 2.0**	57.1 ± 1.8
	51.0	3	27.3 ± 5.0*	18	28	6	7	52	34.7 ± 1.2**	56.4 ± 4.0
Vinclozolin	5.0	3	4.0 ± 2.0	2	5	—	3	7	4.7 ± 2.3	60.7 ± 3.0
	8.5	3	8.0 ± 3.5*	3	10	—	7	13	8.7 ± 3.1*	54.2 ± 2.6
	17.0	3	14.7 ± 4.2*	15	7	—	14	22	14.7 ± 4.2*	47.7 ± 3.5
	51.0	3	21.3 ± 3.1*	9	25	—	10	34	22.7 ± 4.2*	46.5 ± 4.0*
Atrazine	5.0	3	6.0 ± 4.0	3	5	2	4	10	6.7 ± 5.0	48.1 ± 3.9
	8.5	3	8.7 ± 1.2*	4	10	—	7	14	9.3 ± 1.2*	41.4 ± 3.9*
	17.0	3	14.7 ± 3.1*	5	17	—	9	22	14.7 ± 3.1*	34.1 ± 5.9*
	51.0	3	28.0 ± 4.0*	13	25	5	5	43	28.7 ± 3.1*	27.7 ± 4.7*

[a]Percent of total number of cells with at least one chromosomal aberration but not gaps/total number of cells scored.
[b]Percent of total number of aberrations without gaps/total number of cells analyzed.
[c]Percent of cells in mitotic division/1,000 cells examined (see Materials and Methods for details).
* $P < 0.05$, ** $P < 0.01$ vs. control.
SD = standard deviation of three independent experiments, each done on a single subject.

TABLE III. Chromosomal Aberrations in Controls and 72-hr Lymphocyte Cultures Exposed to DPX-E9636

Treatment	Concentration (μM)	No. of subjects	% of aberrant cells[a] (Mean ± SD)	Chromosomal aberrations: Breaks: Chromosome type	Breaks: Chromatid type	Fragments	Gaps	No. of total aberrations	Aberration frequency[b] (Mean ± SD)	Mitotic index[c] (Mean ± SD)
Control	—	3	0.7 ± 1.2			—	3	1	0.7 ± 1.2	75.1 ± 6.7
Ethanol	(0.1%)	3	1.3 ± 2.3	2				2	1.3 ± 2.3	70.7 ± 7.3
DPX-E9636	0.0012	3	5.3 ± 3.1	5	4		9	9	6.0 ±)	55.5 ± 3.5
	0.006	3	12.7 ± 3.1*	9	10	2	10	21	14.0 ± 5*	50.5 ± 6.4
	0.012	3	36.7 ± 9.2*	20	35	—	5	55	36.7 ± 2*	36.1 ± 2.9*
	0.12	3	23.3 ± 3.1*	19	16	—	16	35	23.3 ± 1*	28.0 ± 4.2*

[a]Percent of total number of cells with at least one chromosomal aberration but not gaps/total number of cells scored.
[b]Percent of total number of aberrations without gaps/total number of cells analyzed.
[c]Percent of cells in mitotic division/1,000 cells examined (see Materials and Methods for details).
*$P < 0.05$ vs. control.
SD = standard deviation of three independent experiments, each done on a single subject.

cyte cultures exposed to gliphosate, vinclozolin, and atrazine.

DPX-E9636, used at the same concentrations, was extremely cytotoxic, resulting in a low number of scorable metaphases; however, an increase in the percent of aberrant cells as well as in the aberration frequency, including chromatid breaks, chromosome breaks, and fragments, were observed in the range 0.0012–0.12 μM (Table III).

Our data showed a significant dose-dependent increase in the percent of aberrant cells and chromosome aberrations compared to the controls. However, the lowest dose of all pesticides did not induce any statistically significant clastogenic effect, whereas at the highest dose of DPX-E9636 (0.12 μM) a reduction of CAs was evident. Furthermore, the mitotic index reported in Tables II and III indicated a decreasing trend in cell division index of lymphocyte cultures exposed to the increasing concentrations of all pesticides, showing that atrazine and DPX-E9636 were the more effective in decreasing cell growth.

Tables IV and V show the SCE frequencies obtained

TABLE IV. Number of SCE/cell in Controls and 72-hr Lymphocyte Cultures Exposed to Vinclozolin, Gliphosate, and Atrazine

Treatment	Concentration (μM)	No. of subjects	SCE/cell (Mean ± SD)
Control	—	3	1.9 ± 0.9
DMSO	(0.3%)	3	2.2 ± 1.0
Gliphosate	5.0	3	2.3 ± 0.6
	8.5	3	3.6 ± 0.8*
	17.0	3	5.3 ± 0.3*
	51.0	3	4.9 ± 1.3*
Vinclozolin	5.0	3	3.1 ± 1.0
	8.5	3	3.8 ± 1.4*
	17.0	3	4.5 ± 1.2*
	51.0	3	4.4 ± 1.6*
Atrazine	5.0	3	5.5 ± 1.2*
	8.5	3	6.1 ± 0.9*
	17.0	3	5.0 ± 0.9**
	51.0	3	4.7 ± 1.4*

SCE/cell = total number of SCEs/total number of cells analyzed.
*$P < 0.05$, **$P < 0.01$ vs. control.
SD = standard deviation of three independent experiments, each done on a single subject.

TABLE V. Number of SCE/cell in Controls and 72-hr Lymphocyte Cultures Exposed to DPX-E9636

Treatment	Concentration (μM)	No. of subjects	SCE/cell (Mean ± SD)
Control	—	3	1.9 ± 0.9
Ethanol	(0.1%)	3	2.3 ± 1.5
DPX-E9636	0.0012	3	2.4 ± 0.8
	0.006	3	3.8 ± 0.7*
	0.012	3	5.5 ± 1.4*
	0.12	3	6.5 ± 1.3**

SCE/cell = total number of SCEs/total number of cells analyzed.
*$P < 0.05$, **$P < 0.01$ vs. control.
SD = standard deviation of three independent experiments, each on a single subject.

in the exposed lymphocyte cultures of the same donors' group. An increase of SCE/cell was observed in all treated cultures, but there was no general significant variation as pesticide dose increased. The lowest concentrations did not induce an increase in SCE frequency except for the lymphocyte cultures exposed to atrazine; moreover, at the highest concentration of gliphosate, vinclozolin, and atrazine a slight but not significant reduction was evident.

G6PD Activity

We next analyzed G6PD enzyme activity, since we had already obtained evidence demonstrating that an increased activity of this enzyme was always coupled to a change in the cell redox state [Ursini et al., 1997]. Thus, we exposed aliquots of lymphocytes from the three healthy donors to the same cytogenetically tested pesticide doses. G6PD activity was measured 6 hr after treatment. The results are given in Tables VI and VII the exposure time of the cultures was optimal to determine G6PD variations under oxidative stress conditions [Ursini et al., 1997].

A significant enhancement of G6PD activity was observed in the range 8.5–51 μM for gliphosate and vinclozolin and in the range 5–51 μM and 0.0012–0.012 μM for atrazine and DPX-E9636, respectively. The highest induction of G6PD activity reached 163% and 103% over the control in samples exposed to gliphosate and vinclozolin, while the effect was less evident in atrazine and DPX-E9636-exposed cells (see also Fig. 1). Since elevated pesticide concentrations could be cytotoxic, we also determined the cell viability at the end of the incubation period (6 hr). Results (Table VI and VII) showed an increasing trend of cell death in the exposed cultures, more evident at the higher pesticide doses. We repeated the same experiments in non-PHA-stimulated lymphocytes, in order to assess if there was any PHA interference in the observed effect. In nonstimulated lymphocytes cultures we were unable to detect G6PD induction, since cells became extremely sensitive to the pesticide cytotoxic effect (less than 50% of cells were viable) (data not shown).

To ascertain whether the pesticide exposure involved G6PD as an antioxidant enzyme or as a key member of the pentose phosphate pathway, we exposed stimulated lymphocytes from the same subjects to increasing concentrations of each pesticide in the presence or absence of the antioxidant NAC, which is a GSH precursor. The results (Fig. 1) showed that NAC was able to buffer the pesticide-derived increase of G6PD activity, suggesting that the pesticides tested produced a reduction of the cell GSH content and a consequent pro-oxidant state.

DISCUSSION

In order to have a wider view of the genotoxic ability of the pesticides gliphosate, vinclozolin, and atrazine, and to obtain evidence on the mutagenic potential of the recently introduced DPX-E9636, we studied the incidence of cytogenetic damage and the possible induction of oxidative stress in in vitro cultures of human lymphocytes exposed to the four pesticides. Our results indicate that each pesticide induced a dose-related increase of structural CAs, an increase of SCEs, and a change in the redox state of the cell.

From the data available in the literature, no genotoxic activity of gliphosate has been show in Salmonella and *E. coli* WP-2 reversion assays, in the *Bacillus subtilis* recombination assay, in CHO cell gene mutation test, and in the rat primary hepatocyte/DNA repair assay [Li and Long, 1988], whereas weak positive results have been

TABLE VI. Effect of Vinclozolin, Gliphosate, and Atrazine on G6PD Activity and Cell Viability in 6-hr Exposed Lymphocyte Cultures

Treatment	Concentration (μM)	No. of subjects	G6PD activity U/mg of protein (Mean ± SD)	% of cell killing[a] (Mean ± SD)
Control		3	0.30 ± 0.07	4.85 ± 3.38
DMSO	(0.3%)	3	0.28 ± 0.05	4.92 ± 0.33
Gliphosate	5.0	3	0.31 ± 0.02	6.35 ± 1.79
	8.5	3	0.56 ± 0.02*	7.39 ± 3.24
	17.0	3	0.79 ± 0.05*	10.39 ± 1.56
	51.0	3	0.62 ± 0.06*	13.49 ± 2.97
Vinclozolin	5.0	3	0.34 ± 0.06	5.17 ± 1.36
	8.5	3	0.43 ± 0.03*	6.24 ± 1.38
	17.0	3	0.61 ± 0.04*	8.56 ± 2.62
	51.0	3	0.60 ± 0.02*	12.33 ± 1.12
Atrazine	5.0	3	0.36 ± 0.08*	5.92 ± 1.22
	8.5	3	0.37 ± 0.06*	6.94 ± 1.58
	17.0	3	0.45 ± 0.04*	8.25 ± 2.10
	51.0	3	0.47 ± 0.10*	10.51 ± 2.57

[a]Percent of total number of cell killing/total number of cells.
*$P < 0.05$ vs. control.
SD = standard deviation of three independent experiments, each done on a single subject.

TABLE VII. Effect of DPX-E9636 on G6PD Activity and Cell Viability in 6-hr Exposed Lymphocyte Cultures

Treatment	Concentration (μM)	No. of subjects	G6PD activity U/mg of protein (Mean ± SD)	% of cell killing[a] (Mean ± SD)
Control	—	3	0.30 ± 0.07	4.85 ± 3.38
Ethanol	(0.1%)	3	0.33 ± 0.03	2.63 ± 0.28
DPX-E9636	0.0012	3	0.36 ± 0.06*	3.79 ± 0.11
	0.006	3	0.43 ± 0.04*	4.88 ± 0.87
	0.012	3	0.43 ± 0.03*	6.93 ± 0.80
	0.12	3	0.35 ± 0.05	11.74 ± 0.24

[a]Percent of total number of cell killing/total number of cells.
*$P < 0.05$ vs. control.
SD = standard deviation of three independent experiments, each done on a single subject.

obtained in the SCE assay in human lymphocytes by Vigfusson and Vyse [1980] and by Rank et al. [1993] in mouse bone marrow micronucleus test, in Salmonella mutagenicity assay, and in the Allium anaphase-telophase test. Our results showed a weak induction of SCE frequency and a significant increase of CAs in lymphocyte cultures treated with gliphosate.

Vinclozolin has been studied in different genetic tests: some authors have reported a transforming activity in BALB/c3T3 cells [Perocco et al., 1993] and a mutagenic effect in *Salmonella typhimurium* and in *Schizosaccharomyces pombe* [Chiesara et al., 1982]. No positive results have been obtained either in SCE or CA assays in human lymphocytes after a 24-hr treatment [Hrelia et al., 1996; Kevekordes et al., 1996]. The apparent discrepancy between our data, which indicated a genotoxic effect, and those of Hrelia and Kevekordes may be due to the different exposure time (72 hr) of our experimental system.

Several studies on atrazine have shown that it acts as a mutagenic compound in mouse bone marrow cells in vivo [Adler, 1980], as well as in human lymphocytes in vitro [Ribas et al., 1995]. Our finding that atrazine showed an increase in cytogenetic damage is in agreement with these reports and with Meisner et al. [1992], who observed an induction of CAs in 72-hr human lymphocyte cultures.

DPX-E9636 belongs to the recently introduced class of sulfonylureas, applied at very low concentrations in agriculture (g/ha instead of Kg/ha). Some studies reported the herbicidal activity and the toxicity of sulfonylureas on microbial strains and soil respiration [Dumontet et al., 1993; Murai et al., 1995] but no evidence is available

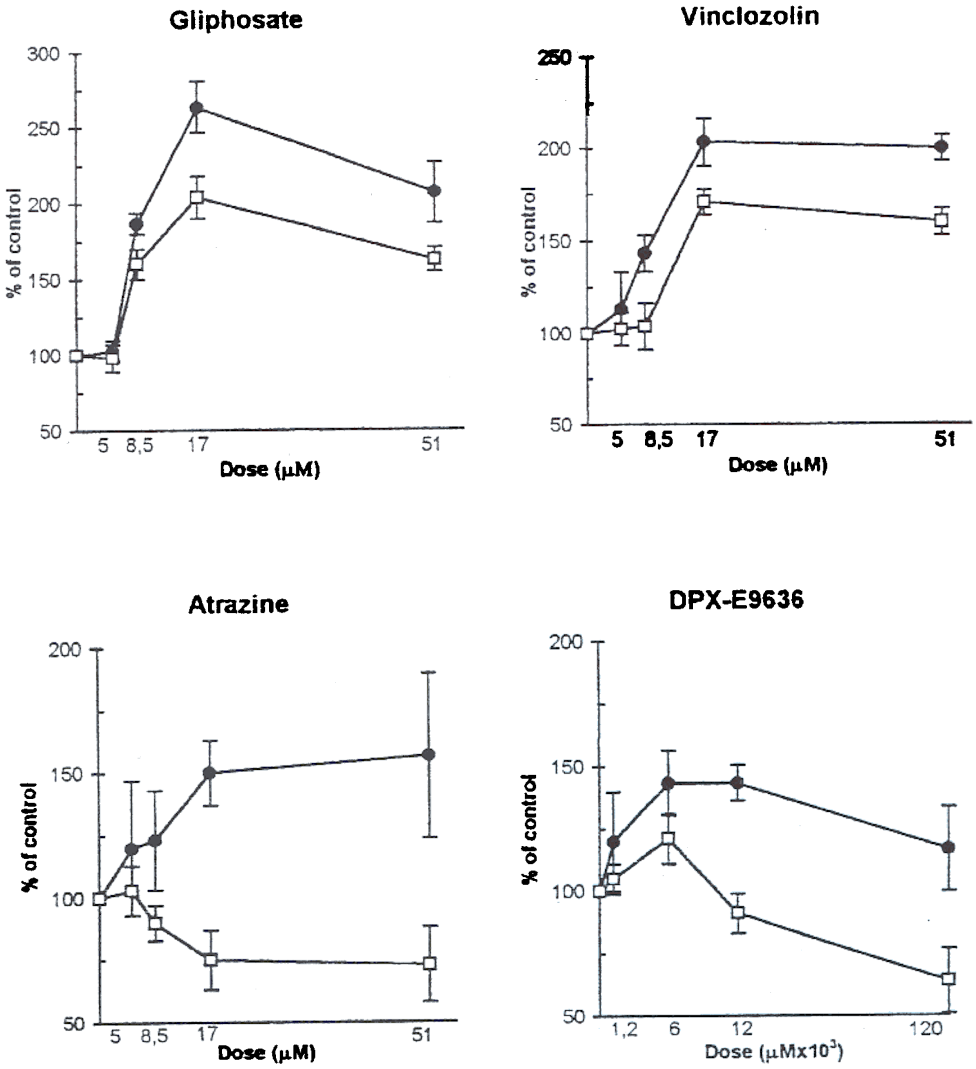

Fig. 1. Reduction of G6PD enzyme activity in both control and pesticide-exposed lymphocyte cultures from three healthy donors, treated (white symbol) or not (black symbol) with the antioxidant N-acetyl-cysteine (NAC) (mean ± SD of three independent experiments, each done on a single subject).

on the genotoxicity of this class of pesticides. In our experimental system, DPX-E9636 induced a significant increase in CAs and SCEs, but at the highest dose tested, we observed a reduction of both percent of aberrant cells and aberration frequency, probably due to the increase of pesticide-dependent cytotoxicity.

On the other hand, we also observed, for each pesticide, a significant increase of SCE/cell, but the curves of SCE induction were quite flat. A slight but not significant reduction of SCE frequency found at the highest concentrations of gliphosate, vinclozolin, and atrazine could be a consequence of treatment with cytotoxic doses, as suggested by the decreasing trend of the mitotic index.

Since we aimed to study the effects of a longer time exposure to pesticides, cytogenetic analysis was carried out on 72-hr lymphocyte cultures. Such a culture time is not commonly considered the best for the detection of CAs, whose frequency may be underestimated as a consequence of the presence of second mitoses. This effect should be compensated for partly by a longer exposure of lymphocytes to the chemical compounds and/or to their metabolites; in any case, it does not compromise the basic conclusion on the genotoxicity of pesticides, as already suggested by De Ferrari et al. [1991] and by Bonatti et al. [1994].

Even though many studies have confirmed the genotoxic potential of several pesticides, little is known about the molecular mechanisms involved in cell response to these chemical agents. In order to detect a possible involvement of oxidative stress, we analyzed G6PD activ-

ity following pesticide exposure in human lymphocyte cultures. We used a 6-hr treatment since we had already demonstrated that in several human cell lines G6PD activity stimulation persists for at least 6 hr after exposure to oxidative agents, but the stimulation decreases with longer incubations [Ursini et al., 1997].

The different but statistically significant enhancement of G6PD activity induced by the pesticides was comparable to the increase we found by treating human lymphocytes with the GSH oxidative agent diamide (data not shown). We observed that the major increase of G6PD activity was obtained in lymphocyte cultures exposed to gliphosate and vinclozolin—both seemed to affect the proliferating ability to a lesser extent. Moreover, at the highest dose of the chemicals G6PD activity remained approximately constant or decreased, whereas the cytotoxic effect increased. This observation could be explained either by G6PD enzyme instability consequent to a hyperoxidative cell condition, as already reported for other proteins [Grune et al., 1997], or by an increased cytotoxic effect. Furthermore, our finding that pesticide treatment induced CAs, SCEs, and resulted in a change in the cell redox state agrees with some reports indicating that reactive oxygen species may be involved in the toxicity produced by other pesticides [Fridovich, 1978; Bagchi et al., 1995; Dahlhaus et al., 1995] and with data obtained in bovine lymphocytes exposed to pesticides [Lioi et al., 1998].

In summary, the results presented in this study indicate that the pesticides tested are genotoxic and support the hypothesis that cells exposed to these biologically active compounds could have altered cell metabolism, probably generating ROIs through a mechanism still unknown. However, rapid depletion of intracellular GSH and subsequent activation of G6PD would be expected to be a primary event after pesticide treatment. Furthermore, continuous exposure to these chemicals (for 72 hr) would result in the inability of HMP shunt to replenish the GSH intracellular pool, and thus to protect the cells against oxidant injury.

Further studies are needed to determine at which steps the pesticides herein tested are acting in the biochemical pathway controlling ROIs production.

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Fundamental and Molecular Mechanisms of Mutagenesis

Genotoxicity and oxidative stress induced by pesticide exposure in bovine lymphocyte cultures in vitro

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Abstract

The genotoxic activity of the pesticides gliphosate, vinclozolin and DPX-E9636 was studied in in vitro cultures of bovine lymphocytes, using chromosome aberration (CA) and sister chromatid exchange (SCE) frequencies as genetic end-points and a variation of glucose 6-phosphate dehydrogenase (G6PD) enzyme activity as a marker of changes in the normal cell redox state. Results indicated a statistically significant increase of structural aberrations, sister chromatid exchanges and G6PD activity, suggesting that the pesticides tested induce either oxidative stress or a mutagenic effect in this species. The evaluation of both mitotic index and cell viability, after pesticide exposure, demonstrates a high cytotoxic effect which is always associated with the observed genotoxic effect.

Keywords: Bovine; Pesticide; Chromosome aberration; Sister chromatid exchange; Oxidative stress; Glucose 6-phosphate dehydrogenase

1. Introduction

Pesticides are chemical compounds which have to comply with two requirements: a high degree of toxicity and a high target specificity.

Even though the presence of pesticide residues has been demonstrated in raw bovine milk and in different farm animal tissues at maximum or higher levels than allowed by the international tolerance levels [1–3], little is known about the possible effects and molecular consequences of pesticide exposure in livestock. It has been reported that the exposure to some pesticides determines both a serious decrease of progesterone secretion in in vitro cultures of bovine granulosa cells from preovulatory follicles [4] and an increase in the number of sister chromatid exchanges (SCEs) in chick embryo cells [5].

In the present study we have analysed the genotoxic potential of the pesticides gliphosate, vinclozolin and the recently introduced DPX-E9636 in in vitro bovine lymphocyte cultures, from three healthy bovines, using as genetic end-points the frequency of chromosomal aberrations (CAs) and of SCEs. We have also evaluated the induction of oxidative stress,

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following pesticide treatment, by measuring G6PD activity, an enzyme catalysing the first and rate-limiting step of the pentose phosphate pathway. G6PD action provides reducing power, in the form of NADPH, which is used by cells to drive enzymatic reactions required to remove reactive oxygen intermediates (ROIs) [6] and necessary to maintain the intracellular content of the prevalent antioxidant molecule present in the cell cytoplasm: reduced glutathione (GSH) [7]. Recent reports have clearly demonstrated that cell oxidative stress conditions and thus GSH depletion, are always followed by increased G6PD activity, indicating that G6PD is functioning as an antioxidant enzyme [8,9]. Furthermore, it has been observed that nucleated cells, bearing genetically determined G6PD null mutation, are extremely sensitive to oxidative stress [10]. We therefore examined both the cytogenetic effects and G6PD enzyme activity in bovine lymphocytes after pesticide exposure.

Our data indicate a mutagenic effect of the tested chemicals in bovine species as well as an induction of oxidative stress and thus a change in the cell redox state.

2. Materials and methods

2.1. Test chemicals

The following chemical agents were tested: gliphosate (*N*-(phosphonomethyl)glicine) and vinclozolin (3-(3,5-dichlorophenyl)-5-methyl-5- vinyloxazolidine-2,4-dione) from Lab Service Analytica (Bologna, Italy) and DPX-E9636 (1-(4,6-dimethoxypyrimidin-2-yl)-3-(ethylsulfonyl-2-pyridysulfonyl)urea) from Du Pont (Paris, France) (purity of all active ingredients $\geq$ 98%). The features of pesticides assayed were indicated in Table 1 as certified by the supplying firms and reported by Worthing and Walker [11] and Perucci et al. [12]. Gliphosate was dissolved in sterile water whereas vinclozolin and DPX-E9636, because of their low water solubility, were dissolved in dimethyl sulfoxide (DMSO, Fluka, Buchs, Switzerland) 0.3% and ethanol (Sigma, St. Louis, MO, USA) 0.1% final concentration in culture, respectively. An equal concentration of DMSO or ethanol-containing culture medium was utilised as solvent positive controls.

2.2. Lymphocyte cultures

Peripheral blood was drawn from the jugular vein of three clinically healthy unrelated cows, nearly two years of age, by using heparinized vacutainers. Lymphocytes were separated by Ficoll–Hypaque gradient density following standard techniques [13]. One milliliter of buffy coat was cultured in 9 ml of RPMI 1640 medium (Dutch modification) supplemented with 15% heat inactivated fetal calf serum (FCS), 10 μg/ml L-glutamine and 10 μg/ml Pokeweed mitogen, all obtained from Gibco (New York, NY, USA). Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C. To eliminate variable sources due to culture conditions, exposed and control cultures were concurrent; the same batches of culture medium and an equal number (1×10^6) of cells/plate were used. For CA and SCE analysis pesticides were added immediately after Pokeweed stimulation and left throughout the whole culture period.

2.3. Cytogenetic assays

Cells were cultured for 72 h at 37°C for CA studies. After 70 h of incubation, colcemid (Gibco, 50 μl for 10 ml cultures) was added and left during the last 2 h of cell growth. Lymphocytes were collected by centrifugation, resuspended in prewarmed hypotonic solution (0.075 M KCl) for 20 min, fixed

Table 1
Pesticides tested

	Molecular formula	CAS registry number	Activity	Field doses	Toxicity in rats, LD_{50} (mg/kg)
		1071-83-6			4320
		50471-44-8			> 10000
		122931-48-0			> 5000

in methanol/acetic acid (3:1) for 10 min and stained with a 5% Giemsa solution (pH 6.8) for 10 min.

For SCE analysis, 30 h prior harvesting, BrdU (Sigma) was added to each culture (10 μg/ml final concentration). Samples were protected from light. Colcemid was added during the final 2 h (50 μl for 10 ml cultures); harvested cells were treated with hypotonic solution and fixed with methanol/acetic acid (3:1). Air dried slides were stained with a 0.2% acridine orange solution in phosphate buffer (pH 6.8) and sealed with paraffin.

From each concentration and from each subject, 50 well spread metaphases bearing 60 chromosomes were scored on blindly coded slides for CAs and for SCEs. Structural aberrations (isochromatid and chromatid breaks, gaps, fragments and chromosomal rearrangements) were classified according to the criteria suggested by Savage [14]. Gaps were reported but not included either in the calculation of aberration frequency or in the percentage of aberrant cells.

The MI was evaluated counting at least 1000 cells per treatment: the number of dividing cells (prophases and metaphases) was divided by the total number of all cells [15].

2.4. G6PD enzyme assay

Mitogen stimulated cells were cultured for 48 h at 37°C in order to analyse a larger number of cells. An equal number of lymphocytes from each subject were plated and, except for the controls, treated for 6 h with increasing concentrations of each pesticide. Cells were collected and centrifuged, G6PD activity was determined, as already described [16], by measuring the rate of increase in absorbance at 340 nm due to the conversion of $NADP^+$ to NADPH. Since 6-phosphogluconate dehydrogenase (6PGD), the second enzyme of the pentose phosphate pathway, also produces NADPH, both 6PGD and total dehydrogenase activity (G6PD plus 6PGD) were determined separately as previously reported, in order to obtain accurate enzyme activity for G6PD [17,18].

Antioxidant *N*-acetyl-cysteine (NAC, Sigma) treatment was performed adding 40 mM final concentration of this substance dissolved in PBS (Gibco) in both exposed and control cultures 1 h prior to pesticide exposure and left during the incubation period [19].

Cell survival was determined by the Trypan-blue (0.5% solution, Serva, Heidelberg, Germany) exclusion technique.

2.5. Statistical analysis

The comparison between the exposed and control groups for each pesticide and for each dose was performed by means of the two-tailed paired Student's *t*-test.

3. Results

3.1. CA, SCE and MI analysis

In order to assess the mutagenic potential of pesticides gliphosate, vinclozolin and DPX-E9636 in in vitro bovine lymphocyte cultures after 72 h exposure, we first carried out a preliminary study to determine the higher doses at which we were still able to observe a sufficient number of metaphases either for CAs or for SCEs. We chose concentrations ranging from 17 to 170 μM for gliphosate and vinclozolin; DPX-E9636 used at the same concentrations was extremely cytotoxic. The fixed number of cells scored were observed in the range 0.0012–12 μM (Table 2).

As shown in Table 2, each chemical compound produced a significant increase in the percentage of aberrant cells as well as in the aberration frequency (isochromatid breaks and chromatid breaks). No fragments or chromosomal rearrangements were found. The genotoxic effect evidenced in our experimental conditions was always associated with a considerable reduction of the mitotic index ($P < 0.05$ for all examined pesticides). These results showed that all tested pesticides induced a significant clastogenic effect and a dose-dependent decreasing trend of cell proliferation, which was mainly evident in DPX-E9636 and vinclozolin exposed lymphocyte cultures.

Table 3 shows the SCE frequencies obtained in pesticide-exposed lymphocyte cultures of the same donors' group. All pesticides induced a dose-dependent increase in the number of SCE/cell in exposed cultures compared to controls; furthermore, at the highest concentrations a slight but not significant reduction was evident.

Table 2
Chromosomal aberrations in controls and 72 h pesticide-exposed bovine lymphocyte cultures

Treatment	Dose (μM)	No. of subjects	No. of cells scored	Chromosomal aberrations			Aberration frequency[a]	% of cells with aberrations[b]	Mitotic index[c]
				Isochromatid breaks	Chromatid breaks	Gaps		MV ± SD	MV ± SD
Control	–	3	150	2	5	1	4.7 ± 1.2		
DMSO	(0.3%)	3	150	2	3	1	3.3 ± 1.2		
Ethanol	(0.1%)	3	150	2	6	1	5.3 ± 1.2		
Gliphosate	17	3	150	11	18	3	19.3 ± 4.2 *		
	85	3	150	22	25	6	31.3 ± 6.1 *		
	170	3	150	17	41	5	38.7 ± 7.0 *		
Vinclozolin	17	3	150	18	27	8	30.0 ± 5.3 *		
	85	3	150	16	42	9	38.7 ± 3.1 * *		
	170	3	150	28	54	12	54.7 ± 2.3 * *		
DPX-E9636	0.012	3	150	20	25	10	30.0 ± 3.5 *	24.0 ± 5.3 *	6.4 ± 0.5 *
	0.12	3	150	20	32	9	34.7 ± 7.6 *	28.7 ± 6.1 *	4.8 ± 0.3 *
	12	3	150	22	34	0	37.3 ± 10.3 *	29.3 ± 3.1 *	3.2 ± 0.3 *

[a] Total number of aberrations without gaps/total number of cells analysed, × 100.
[b] Total number of cells with at least one chromosome aberration but not gaps/total number of cells scored, × 100.
[c] Cells in mitotic division/1000 cells examined, × 100.
* $P < 0.05$, * * $P < 0.01$ vs. control.
MV ± SD: mean value ± standard deviation.

Table 3
Number of sister chromatid exchanges per cell (SCE/cell) in controls and 72 h pesticide-exposed bovine lymphocyte cultures

Treatment	Dose (μM)	No. of subjects	No. of cells scored	SCE/cell (MV ± SD)
	–			
	(0.3%)			
	(0.1%)			
	17			
	85			
	170			
Vinclozolin	17			
	85			
	170			
DPX-E9636	0.012			
	0.12			
	12			

SCE/cell: total number of SCEs/total number of cells scored.
* $P < 0.05$ vs. control.
MV ± SD: mean value ± standard deviation.

3.2. G6PD activity

To study the possible induction of oxidative stress following pesticide treatment, we analysed G6PD activity in the three bovine subjects exposed to the same pesticides, since we have already obtained evidence demonstrating that an increased activity of this enzyme is always coupled to the pro-oxidant state of the cell [9]. Thus we exposed aliquots of lymphocyte cultures to the same cytogenetically-tested pesticide doses. G6PD activity was measured 6 h after treatment. Results are reported in Table 4; the exposure time chosen was optimal to determine G6PD variations under oxidative stress conditions [9].

A significant enhancement of G6PD activity was observed in all treated lymphocyte cultures compared to the controls. The highest induction of G6PD stimulation reached 118% and 89% over the control, in samples exposed to the lower concentrations of gliphosate and vinclozolin, while the effect was less evident at the highest dose of DPX-E9636 (see also Fig. 1).

Since elevated pesticide concentrations could be cytotoxic for the cells we also determined the percentage of cell killing at the end of the incubation period (6 h). Results (Table 4) showed an increasing but not statistically significant cytotoxicity in the exposed cultures.

We repeated the same experiments in non (Pokeweed) stimulated lymphocytes, in order to assess if there was any mitogen interference in the observed effect. In non-stimulated lymphocyte cultures we were unable to detect G6PD induction, since cells became extremely sensitive to the cytotoxic effects of the pesticide (more than 60% of cells were killed) (data not shown).

To ascertain whether the pesticide exposure involved G6PD as an antioxidant enzyme or as a key member of the hexose monophosphate shunt, we exposed stimulated lymphocytes from the subjects to

Table 4
Effects of pesticide exposure on G6PD enzyme activity and cel death in controls and 6 h treated bovine lymphocyte cultures

Treatment	Dose (μM)	G6PD activity[a]	% of cell killing[b]
		MV ± SD	MV ± SD
Control	–		
DMSO	(0.3%)		
Ethanol	(0.1%)		
Gliphosate	17		
	85		
	170		
Vinclozolin	17		
	85		
	170		
DPX-E9636	0.012		
	0.12		
	12		

[a] U/mg of protein.
[b] Total number of cells killed/total number of cells, × 100
[c] $P < 0.05$ vs. control.
MV ± SD: mean value ± standard deviation.

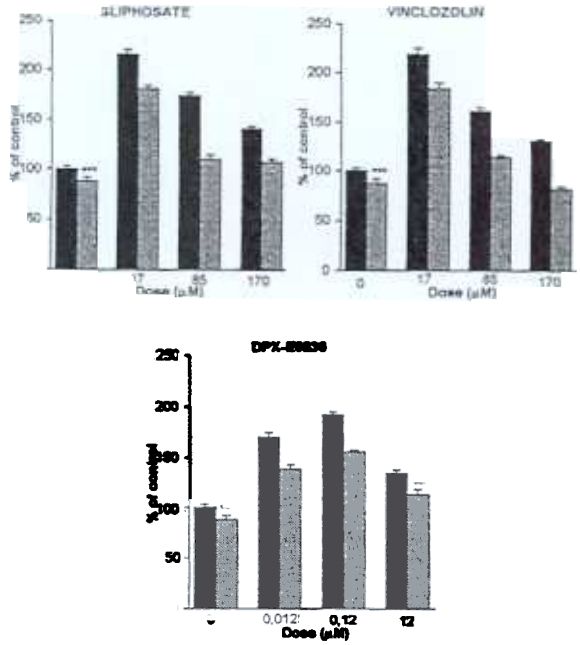

Fig. 1. G6PD enzyme activity in controls and 6 h pesticide-exposed lymphocyte cultures from three bovine subjects in the absence (black graph) or in the presence (bar graph) of the antioxidant NAC. (* * *) Not significant mean value of G6PD activity compared to the mean value obtained in the absence of NAC.

the same increasing concentrations of pesticides in the presence or in the absence of the antioxidant NAC, which is a GSH precursor. Results reported in Fig. 1 show that NAC buffers the pesticide-derived increase of G6PD activity thus indicating that the chemicals produced a reduction of intracellular GSH pool and a consequent pro-oxidant state.

4. Discussion

The study of the genotoxicity of environmental mutagens in domestic animals is of particular concern because of their possible consequences on the productive and reproductive efficiency of livestock. In the past, risk assessment was limited to the danger to human health, but nowadays ecological risks are also taken into account. In this sense, further studies are required in order to determine the mechanism of action of pesticides, how they interfere with metabolism, genetic damage and detoxification mechanisms in domestic animals. From this point of view, the literature is quite scanty: the only available data on the chemicals herein tested are reported in human or in bacterial mutagenicity assays [20,21] but no specific effects are demonstrated in bovine or in other domestic species.

The present study provides evidence for the intrinsic mutagenic potential of the herbicides gliphosate and DPX-E9636 and of the fungicide vinclozolin on in vitro cultures of bovine lymphocytes from three subjects randomly chosen. Our results indicate a statistically significant increase of both structural CAs and SCEs in exposed cultures compared to the controls. The slight reduction of SCEs, observed at the highest pesticide concentration, could be a consequence of the treatment with highly cytotoxic doses. This observation is in agreement with the lower mitotic index found at these doses. Moreover, all doses tested of each pesticide produced cytogenetic damage and a change in the cell redox state; this finding corroborates the recently reported evidence that reactive oxygen species may be involved in pesticide toxicity [22,23].

In order to increase our knowledge about the genotoxicity of the tested environmental mutagens and to gain insight into their mechanism of action, we also analysed the change of the redox state of the cell following pesticide treatment by evaluating G6PD enzyme activity. We chose to detect the pesticides' effect on G6PD activity after 6 h treatment as we had already demonstrated that, in eukaryotic cell lines, G6PD activity stimulation persists for at least 6 h after the addition of oxidative agents, but that the stimulation decreases with longer incubation periods [9].

We observed a different but statistically significant enhancement of G6PD activity, induced by pesticides, comparable to the increase we found by treating bovine lymphocytes with the GSH oxidative agent, diamide (data not shown). The maximum enhancement of G6PD activity was reached at the lowest doses of gliphosate and vinclozolin and at the intermediate concentration of DPX-E9636. The observation that at the higher doses of the chemicals, G6PD activity decreases could be explained by G6PD enzyme instability consequent to an hyper oxidative cell condition, as already reported for other proteins [24]. Moreover, as dose of vinclozolin or DPX-E9636

increases, besides the decrease of G6PD activity, we also observed an increasing cytotoxic effect. This finding would indicate a threshold over which the normal cell circuits could be altered as well as cells becoming more sensitive to pesticide dependent cytotoxicity. The cytogenetic damage and the induction of pro-oxidant state of the cell seems to be a general response as we witnessed the same phenomenon in lymphocyte cultures exposed to atrazine, another chemical agent widely used in agriculture (data not shown).

In summary, our results indicate that the tested pesticides are genotoxic in in vitro cultures of bovine lymphocytes and support the hypothesis that cells exposed to these chemical compounds have altered cell metabolism, probably generating ROIs through a mechanism still unknown. However, rapid depletion of intracellular GSH and subsequent activation of G6PD would be expected to be a primary event after pesticide treatment. Furthermore, continuous exposure to these chemicals (for 72 h) would result in the inability of the hexose monophosphate shunt to replenish GSH intracellular pool and thus to protect the cells against oxidant injury. The evidence reported here has been strengthened by experiments carried out in human lymphocytes in which the pesticide induced genotoxicity resulted in a change in the cell redox state (manuscript in preparation).

Further studies are necessary in this research field as the presence of organic compounds such as pesticides in animal diets may affect not only the health of livestock themselves but also the qualitative and/or quantitative characteristics of animal productions.

Acknowledgements

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Oxidative liver DNA damage in rats treated with pesticide mixtures

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Abstract

Oxidative damage was quantified in the liver of rats by measuring the levels of 8-OH-2-deoxyguanosine (8-OH-2DG) relative to 2-deoxyguanosine in DNA after treating rats for 10 days at a total dose of 1 mg/kg/day with a mixture of the 15 pesticides most commonly found in Italian foods (comprised of dithiocarbamate, benomyl, procymidone, methidathion, chlorpyrifos-ethyl, parathion-methyl, chlorpropham, parathion, vinclozolin, chlorfenvinphos, pirimiphos ethyl, thiabendazole, fenarimol, diphenylamine and chlorothalonil). We fractionated this pesticide mixture into subgroups in order to determine which molecules, if any, induced DNA oxidative damage. The administration of diphenylamine (0.09–1.4 mg/kg/day) and chlorothalonil (0.13–1 mg/kg/day) induced a dose-dependent increase in 8-OH-2DG levels in liver DNA. The other 13 pesticides of the mixture on the contrary, did not produce oxidative liver DNA damage. These results indicate that the toxicity of low doses of pesticide mixtures present in food might be further reduced by eliminating diphenylamine and chlorothalonil. Copyright © 1997 Elsevier Science Ireland Ltd.

Keywords: Pesticides; Oxidative DNA damage; 8-OH-2-deoxyguanosine

1. Introduction

We previously demonstrated that of all the pesticides detected in Italian foods, only 15 are commonly encountered in sizable concentrations (Dolara et al., 1993). By using data on food intake for a normal Italian adult and the mean concentration of these pesticides in food, we calculated the average human exposure through the diet. We then carried out a series of experiments

Abbreviations: Mix, mixture of fifteen pesticides; 8-OH-2-DG, 8-OH-2-deoxyguanosine; 2DG, 2-deoxyguanosine; MN, micronuclei; SCE, sister chromatid exchange; n-SCS, non-synchronous centromeric separation; THIA, thiabendazole; FEN, fenarimol; DIPH, diphenylamine; CHLO, chlorothalonil.

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to determine the toxicity of these 15 pesticides, using a mixture (Mix) in which the single pesticide concentration was set at a level representing the average exposure to each compound through food (Dolara et al., 1993). The composition of the Mix is specified in Table 1. The Mix did not increase the frequency of micronuclei (MN) in rat bone marrow in vivo; in human lymphocytes in vitro it did not vary the frequency of sister chromatid exchange (SCE). On the contrary, the number of non-synchronous centromeric separations (n-SCS) was increased in human lymphocytes in vitro, an effect due to the presence of benomyl (Dolara et al., 1994). We later reported that the administration of the Mix for 10 days increased 8-OH-2-deoxyguanosine (8-OH-2DG), a marker of oxidative damage, in rat liver DNA (Lodovici et al., 1994). These last findings indicated that one or more components of the Mix were able to induce oxidative damage through the production of free radicals.

Since free radicals are supposed to be involved in aging and carcinogenesis (Ames, 1989), we wanted to find which of the pesticides, if any, induced oxidative damage in vivo.

For selected compounds, such as fenarimol (Grabarczyk and Kopec Szlezak, 1992) and methylparathion (Yang et al., 1992), a mechanism involving free radicals has been suggested to account for some toxic effects in vitro (Grabarczyk and Kopec Szlezak, 1992). However, direct evidence of the production of free radicals from pesticides in vivo is lacking in the literature and on this premise we decided to further study the effects of these compounds administered orally to rats, to determine which pesticides could determine oxidative DNA damage.

2. Materials and methods

2.1. Chemicals and standards

All pesticides were obtained from Lab Service (Bologna, Italy); chlorfenvinphos and dithiocarbamate were 95 and 86% pure, respectively, while the others were 99.9% pure. For 8-OH-2DG, we prepared a chromatographic standard as reported in a previous paper (Lodovici et al., 1994).

2.2. Pesticide mixtures

We treated animals with three subgroups of the original Mix, maintaining the same ratios of the different compounds relative to the total dose administered in previous experiments (Lodovici et al., 1994).

We, therefore, employed the following pesticide subgroups:

Mix 1: dithiocarbamate (0.207 mg/kg/day) + benomyl (0.196 mg/kg/day);

Mix 2: procymidone (0.08 mg/kg/day) + methidathion (0.023 mg/kg/day) + chlorpyrifos-ethyl (0.02 mg/kg/day) + parathion-methyl (0.01 mg/kg/day) + chlorpropham (0.007 mg/kg/day) + parathion (0.007 mg/kg/day) + vinclozolin (0.003 mg/kg/day) + chlorfenvinphos (0.003 mg/kg/day) + pirimiphos-ethyl (0.001 mg/kg/day);

Mix 3: thiabendazole (THIA) (0.149 mg/kg/day) + fenarimol (FEN) (0.02 mg/kg/day) + diphenylamine (DIPH) (0.140 mg/kg/day) + chlorothalonil (CHLO) (0.130 mg/kg/day).

Table 1
Calculated average pesticide residues in the normal adult diet of central Italy

Pesticide	Residue (μg/diet)	Percentage of total
	148	20.7
	140	19.6
	107	14.9
	102	14.4
	94	13.1
	57	8.0
	17	2.3
	15	2.0
	14	.9
	7	.0
	5	0.7
	5	0.7
	2	0.3
	2	0.3
		0.1
	716	100%

We also treated four groups of animals with single components of Mix 3. DIPH and CHLO were also administered at different doses in order to construct dose-response curves.

2.3. Animals

Male Wistar rats (200–250 g) were purchased from Morini (Modena, Italy). The animals were treated for 10 consecutive days at 10 a.m. per os with 1 ml corn oil dissolving different mixtures, at doses equivalent to those at which the maximum effect on oxidative damage was previously observed (Lodovici et al., 1994). Controls received corn oil alone. After having identified the active mixtures, experiments were carried out with individual components. No mortality was recorded during the administration of pesticides. Rats were sacrificed by decapitation under a light ether anesthesia, their livers were excised, rinsed in ice-cold 0.15 M KCl and stored at −80°C until analysis.

2.4. Preparation of liver DNA

Isolation and hydrolysis of liver DNA were performed using a published method with a few modifications (Fiala et al., 1989). Briefly, livers (1 g w/w) were homogenized in eight volumes of ice-cold 0.15 M NaCl/0.015 M sodium citrate buffer pH 7 and centrifuged at 1500 × *g* for 15 min. The pellet thus obtained was first solubilized in eight volumes of a pH 7 buffer containing 0.01 M Tris–HCl, 0.001 M EDTA, 1 M NaCl and finally lysed at 0°C for 45 min by the addition of 10% sodium dodecyl sulfate (0.07% of the final concentration). The lysed mixture was extracted with chloroform/isoamyl alcohol (10:2 v/v) and DNA was precipitated from the aqueous phase as reported by Fiala et al. (1989). DNA was then dried with a nitrogen stream, solubilized in 1 ml of 20 mM acetate buffer pH 4.8 and denatured at 90°C for 3 min.

To a 150 μl aliquot of DNA 20 μl of P1 nuclease (5 IU) were added and incubated at 37°C for 1 h in the dark. The incubation mixture was then digested for 1 h at 37°C with 10 μl alkaline phosphatase (3 IU) in the presence of 80 μl of 1

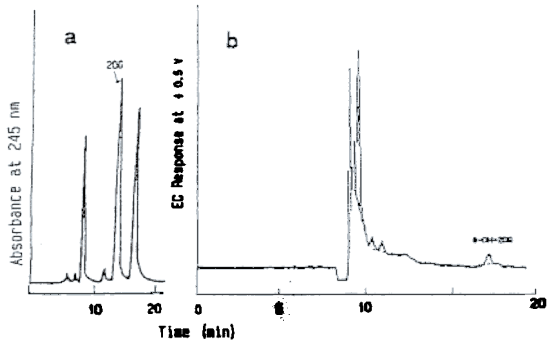

Fig. 1. Typical HPLC chromatograms of hydrolyzed control rat liver DNA. (a) UV detector; (b) Coulochem detector

M Tris–HCl buffer pH 7.8. All samples were protected from light with aluminum foil, in order to avoid artifactual oxidation. The hydrolyzed mixture was finally centrifuged and 70–80 μl of the supernatant were injected into the HPLC.

2.5. HPLC analysis

The separation of 8-OH-2DG and 2-deoxiguanosine (2DG) was performed with an LC/9A Shimadzu HPLC pump using two detectors: UV (Biorad) and Coulochem (ESA model 5100 with a 5010 analytical cell). For chromatographic separation we used two C18 reverse-phase columns in series (Supelco, 5 μm, I.D. 0.46 × 25 cm); the eluting solution was H_2O/CH_3OH (85:15 v/v) with 50 mM KH_2PO_4 pH 5.5 at a flow rate of 0.68 ml/min. The potentials set for electrodes 1 and 2 of the electrochemical detector were 0.02 and 0.5 V, respectively; the UV detector was set at 245 nm. The detectors were connected to a Shimadzu integrator for the determination of peak areas. The retention time for 2DG was about 12.5 min and 18.4 min for 8-OH-2DG as reported in Fig. 1. The detection limit for 8-OH-2DG was 20 pg. The levels of 8-OH-2DG were expressed as the ratio 8-OH-2DG relative to 10^5 2DG bases.

2.6. Statistics

Data were analyzed with the Statgraphics statistical package using one-way analysis of variance and linear regression analysis.

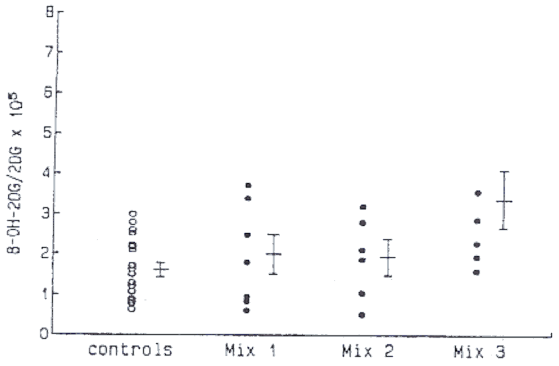

Fig. 2. 8-OH-2DG levels in liver DNA of control rats ($n = 17$) and in rats treated for 10 days with Mix 1 ($n = 7$), Mix 2 ($n = 6$) and Mix 3 ($n = 7$). The composition of the different Mix is specified in Materials and method (Section 2). Individual values and means ± S.E. $^*P < 0.05$.

3. Results

A typical chromatographic profile is shown in Fig. 1. Liver DNA levels of 8-OH-2DG were increased after treatment with Mix 3 ($P < 0.05$) but not after Mix 1 and Mix 2 (Fig. 2). We observed a relatively large inter-individual variability in agreement with results by other authors (Fiala et al., 1989). Since Mix 3 contained THIA, FEN, DIPH and CHLO, we tested each of these pesticides individually at the same dose of the mixtures. Fig. 3 shows that THIA or FEN did not alter the hepatic DNA levels of 8-OH-2DG, whereas DIPH or CHLO increased it relative to controls ($P < 0.05$). These effects were dose-related both for DIPH and CHLO, as shown in Figs. 4 and 5.

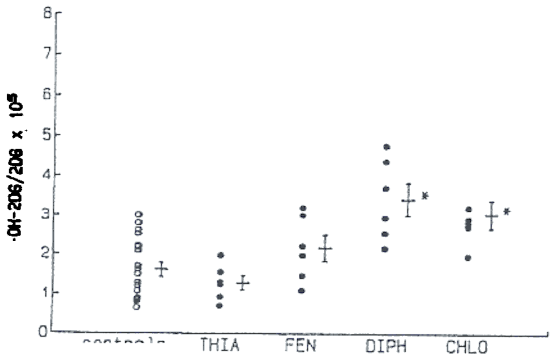

Fig. 3. 8-OH-2DG levels in liver DNA of control rats ($n = 17$) and rats treated for 10 days with 0.13 mg/kg/days of THIA ($n = 6$) and 0.020 mg/kg/days of FEN ($n = 6$); 0.14 mg/kg/days of DIPH ($n = 6$) or 0.13 mg/kg/days of CHLO ($n = 6$). Individual values and means ± S.E. $^*P < 0.05$.

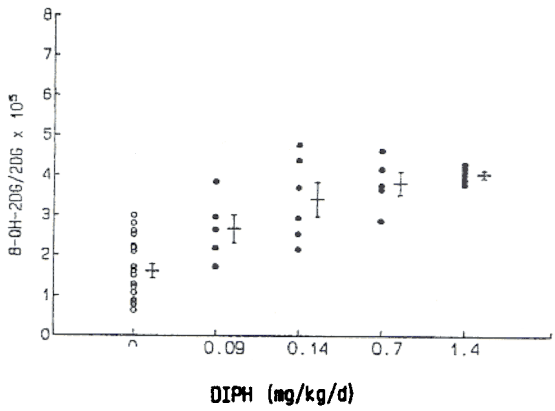

Fig. 4. 8-OH-2DG levels in liver DNA of control rats ($n = 17$) and rats treated with four different doses of DIPH for 10 days. For each dose $n = 6$; $r = 0.72$, $P < 0.001$.

4. Discussion

The formation of 8-OH-2DG from 2DG involves an attack on DNA by reactive oxygen species, probably hydroxyl radicals (Floyd et al.,

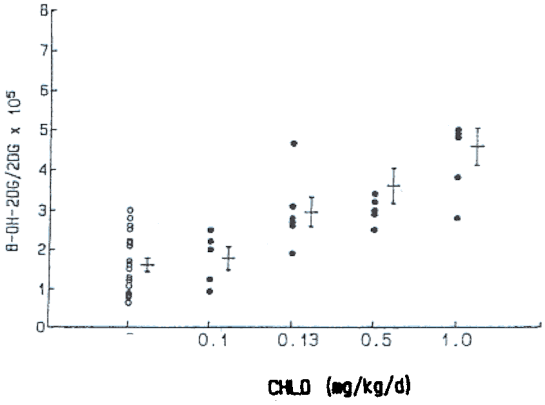

Fig. 5. 8-OH-2DG levels in liver DNA of control rats ($n = 17$) and rats treated with four different doses of CHLO for 10 days. For each dose $n = 6$, except 0.1 mg/kg/days ($n = 5$); $r = 0.81$, $P < 0.001$.

1986). Thus, the increase in the levels of 8-OH-2DG in liver DNA of rats treated with DIPH or CHLO indicates that these pesticides generate reactive oxygen species capable of inducing cell genetic damage. The oxidative damage observed after DIPH administration is in agreement with previous data from the literature (Appel et al., 1987; Valvis et al., 1990). Electron spin resonance spectroscopy has shown that diphenylhydroxylamine, one of the metabolites of DIPH, produces a diphenylnitroxide radical when incubated in vitro with rat and hog liver microsomes (Appel et al., 1987; Valvis et al., 1990). Moreover, dimethylsulfoxide, a hydroxyl radical scavenger (Brayton, 1986), reduces the incidence of DIPH-induced renal papillary necrosis in Syrian hamsters (Lenz and Carlton, 1991), an effect likely mediated by free radical formation. In conclusion, DIPH is a free radical-producing compound in vivo although it appears to be mutagenic in *Salmonella typhimurium* TA98 only in the presence of norharman (Wakabayashi et al., 1982) and is classified as non-carcinogenic for humans (Das et al., 1993).

Our data on liver DNA oxidative damage by CHLO are also consistent with previous results by Yamano and Morita (1995), who demonstrated CHLO-induced lipid peroxidation in rat hepatocytes in vitro (Yamano and Morita, 1995). CHLO can also induce adenomas and carcinomas in rat kidney renal epithelium (NCI, 1978), and has been classified as a human carcinogen with intermediate potency (National Academy of Science, 1987). Although CHLO is considered a carcinogen, it has no genotoxic effects in *Drosophila* (Yoon et al., 1985), in yeast (De Bertoldi et al., 1980), but is mutagenic in the L5178Y tk+/− mouse lymphoma cell line (Davies, 1988). In the case of CHLO it seems reasonable to assume, therefore, that oxidative damage may contribute to the mutagenicity and carcinogenicity of this compound. In fact, the formation of 8-OH-2DG causes misreplication of DNA that may lead to mutation or cancer (Floyd et al., 1986; Shibutani et al., 1991).

In our experiments, the pesticides of Mix 1 and Mix 2 had no effect on oxidative DNA damage, although Mix 2 contained fenarimol and methylparathion, two compounds for which a toxic mechanism involving free radical formation has been hypothesized in vitro (Grabarczyk and Kopec Szlezak, 1992; Yang et al., 1992).

The minimum doses at which DNA oxidative damage is observed with DIPH and CHLO in our experiments were 0.13 and 0.09 mg/kg/day, respectively, about 1/100 the calculated human exposure through food in Italy. Although their concentration is relatively low, the exclusion of these two compounds from use on crops destined for human consumption would reduce the risk of inducing DNA oxidative damage through ingestion of pesticide residues in foods.

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In Vitro Cytotoxic and Cell Transforming Activities Exerted by the Pesticides Cyanazine, Dithianon, Diflubenzuron, Procymidone, and Vinclozolin on BALB/c 3T3 Cells

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Cytotoxic and cell transforming activities of the pesticides cyanazine, diflubenzuron, dithianon, procymidone, and vinclozolin were investigated in vitro by utilizing the BALB/c 3T3 cell transformation test performed in the presence or in the absence of S-9 mix as an exogenous bioactivation system for the chemicals. All the assayed pesticides were cytotoxic in the absence of S-9 mix, whereas only dithianon exerted cytotoxic effects in the presence of metabolic activation. All the chemicals tested did induce BALB/c 3T3 cell transformation, to a various extent, in the absence of S-9 mix. Cell transforming ability of cyanazine and diflubenzuron was not detectable in the presence of S-9.

Key words: cell transformation, environmental risk, multistep carcinogenesis, cell cultures, pesticides metabolism

INTRODUCTION

Few and often inconclusive facts on the possible carcinogenic risk of five pesticides—the fungicides dithianon, procymidone and vinclozolin, the herbicide cyanazine, and the insecticide diflubenzuron—are available in the literature. Cyanazine [EPA, 1985b] and diflubenzuron [EPA, 1985a] are not carcinogenic in rodents; controversial data exist on the oncogenic potential of procymidone [EPA, 1990]; and no data are available on dithianon and vinclozolin.

Different results have been obtained from the short-term tests of mutagenicity. Experiments on cyanazine each time have given negative [Lusby et al., 1979; Moriya et al., 1983], controversial [Plewa et al., 1984], or positive [Ahmed and Grant, 1972; Murnik and Nash, 1977] results. Negative results have been obtained on dithianon [Shirasu et al., 1976] and on diflubenzuron and its metabolites [FAO/WHO, 1981; Moriya et al., 1983]. Procymidone has tested as mutagenic [Georgopoulos et al., 1979], whereas controversial data have been obtained by Chiesara et al. [1982] on vinclozolin in different experimental systems. Also, in a previous study on genotoxicity of these five pesticides, we have obtained negative results when testing sister chromatid exchanges (SCEs) and unscheduled DNA synthesis (UDS, DNA repair) in human peripheral blood lymphocytes (personal observations).

In this study, further data are reported on the in vitro cell transforming potential on BALB/c 3T3 cells of these chemicals, with or without metabolic activation. The aim of the present study was to improve knowledge on the possible carcinogenic risk to humans of these five pesticides. Indeed, in vitro cell transformation is more closely related to in vivo carcinogenesis—69–85% correlation, according to IARC/NCI/EPA Working Group [1985]—than most of the short-term assays.

MATERIALS AND METHODS

Chemicals

The pesticides utilized in the present study, reported in Table I, were purchased from Promochem GmbH (Wesel, Germany). Benzo(a)pyrene (B(a)P) was provided by Sigma Chemical Co. (St. Louis, MO), and 3-methylcholanthrene (3-MCA) was from Fluka Chemicals (Buchs, Switzerland). Pesticides, B(a)P and 3-MCA were dissolved in dimethylsulfoxide (DMSO) (Riedel-De Haën, Hannover, Germany) because of their low water solubility, and 0.5% DMSO-containing medium (final concentration) was utilized in chemical-treated and control cultures.

Cell Cultures

The original stock of BALB/c 3T3 cells, clone A31, was obtained from American Type Culture Collection (Mary-

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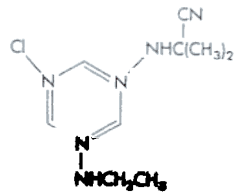
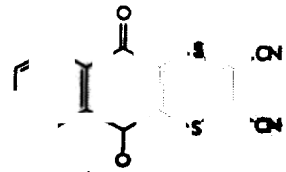
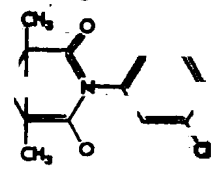
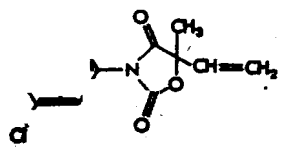
TABLE I. List of Pesticides Assayed in the Study

Common name	Chemical name	Chemical structure	CAS number	Activity[a]	Purity (%)
	2-(4-chloro-6-ethylamino-1,3,5-triazine-2-ylamino)-amino-2-methylpropionitrile		21725–46–2	H	99.5
Diflubenzuron	1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea		35367–38–5		99.9
Dithianon	5,10-dihydro-5,10-dioxonaphto-(2,3-b)-p-dithia-2,3-dicarbonitrile		3347–22–6	F	99.9
Procymidone	N-(3,5-dichlorophenyl)-1,2-dimethyl cyclopropanedicarboximide		32809–16–8	F	99.9
Vinclozolin	3-(3,5-dichlorophenyl)-5-methyl-5-vinyloxozolidine 2,4-dione		50471–44–8	F	96.8

[a]H = herbicide; I = insecticide; F = fungicide.

land). Working cultures were expanded from the original cryopreserved stock. Cells were grown in Dulbecco's modified Eagle's minimal essential medium (D-MEM, Gibco), supplemented with 10% newborn calf serum. The antibiotics streptomycin and penicillin were present at 50 μg/ml and 50 units/ml, respectively. Cells were routinely tested for mycoplasma contamination by the method of Schmidt et al. [1984] and found infection free. Only subconfluent (60–70% confluent) cultures were used in the assay, and the target cells used in the transformation test were not maintained beyond the third passage after thawing.

Preparation of S9 Fraction and Metabolic Activation

S-9 fraction was obtained following the experimental procedure previously reported [Colacci et al., 1990] and essentially based on the method by Ames et al. [1975] from adult male inbred Wistar rats pretreated with phenobarbitone (1 g/liter in the drinking water) during the 6 days before the sacrifice. The amount of each component of the activating system was lowered with respect to that reported in the recommended protocol [Schechtman, 1985] in order to reduce toxic effects on target cells observed in preliminary experiments. Thus the final concentrations of activating system mixture components were: 50 μl/ml S9 fraction, 2 μl/ml glucose-6-phosphate dehydrogenase, 0.2 mM glucose-6-phosphate, 0.28 mM NADH, 0.25 mM NADP, and 0.27 mM NADPH.

Cytotoxicity Test

Cells in exponential growth phase at the density of 3×10^2/60 mm culture dishes (four dishes/treatment) were incubated for 24 hr at 37°C in 5% CO_2 humidified atmosphere. Three concentrations of each chemical, dissolved in 0.5% DMSO, were diluted in culture medium, whereas 0.5% DMSO was added to control cultures. Thereafter, cell cultures were maintained in the presence of the chemicals for 72 hr without S-9 mix (in 10% serum-containing medium). In the modified experimental procedure performed in the presence of S-9 mix cells (in serum-free medium) were exposed to the chemical for 4 hr. After the exposure time, medium was removed and cell cultures were rinsed with phosphate-buffered saline and refilled with fresh newborn calf serum-containing medium. Culture medium was

changed twice a week for the duration of the assay. After 7–10 days, cell cultures were fixed with methanol, stained with 10% Giemsa, and colonies containing more than 50 cells were scored and counted.

Transformation Test

Culture conditions and treatments were performed as described in the cytotoxicity test. Cells in exponential growth phase at the density of 1×10^4/plate, in 10 replicates for each treatment, were exposed to the chemicals. Subtoxic or nontoxic doses of each pesticide were chosen according to the results obtained in cytotoxicity assays and tested for transforming potential. Untreated and DMSO-treated cultures were used as negative controls. Positive controls were performed by utilizing 3-MCA or B(a)P as transforming agents in the absence or in the presence of the S-9 mix activating system, respectively.

Two different experiments were performed following the methods previously described [Schechtman, 1985; Colacci et al., 1990]. The first assay was carried out in order to define the standard parameters of experimental procedure. Cells were exposed to the different pesticides in the absence of S-9 mix-induced bioactivation. In the second experiment cells were exposed to the assayed pesticides for 72 hr in the absence of exogenous bioactivation and 4 hr in the presence of S9-mix. After 2 weeks a level II amplification test was performed by pooling the contents of five confluent plates and reseeding cells at 1×10^5 cells/culture plate in 10 replicates for each treatment.

Plates from level-I and level-II transformations were fixed with methanol, stained with 10% aqueous Giemsa, and scored for the presence of transformation foci 45 days after the treatment.

Scoring of Foci

Only positive foci (type III) were scored according to Schechtman [1985] and to IARC/NCI/EPA Working Group [1985]. Transformation frequencies were assessed by calculating: (1) the total number of foci per group of scored plates, (2) the average number of foci/plate/each treatment ± standard error (SE), and (3) the transformation frequency (TF), i.e., the total number of foci obtained in each treatment per number of cells at risk (surviving cells after each treatment estimated by the clonal efficiency observed in the cytotoxicity assay) [Colacci et al., 1990]. The TF index is a more reliable expression of the real cell transforming activity, since it takes into account the cell clonal efficiency.

Statistical Analysis

Clonal efficiency after exposure to the chemical was analyzed by the Student's t-test. The significance of the difference between the number of foci obtained in each chemical-treated group and that detected in the negative control (0.5% DMSO-treated cultures) was assessed according to the distribution-free Mann-Whitney unpaired t-test.

TABLE II. Cytotoxic Effects of Three Doses of Five Pesticides Dissolved in 0.5% DMSO (final concentration on BALB/c 3T3 Cells Cultured In Vitro†

Treatment	Dose (μg/m	Clonal efficienc (CE)[a]	Relative clonal efficiency (%)[b]
Untreated		0.075	112
DMSO (0.5%)		0.066	100
Cyanazine	10	0.050	75*
Cyanazine	50	0.041	62*
Cyanazine	100	0.013	20**
Diflubenzuron		0.051	77ns
Diflubenzuron	50	0.065	98ns
Diflubenzuron	100	0.038	57**
Dithianon	5	0.060	90ns
Dithianon	25	0.070	106ns
Dithianon	50	0.033	50*
Procymidone		0.060	90ns
Procymidone	50	0.064	96ns
Procymidone	100	0.049	74*
Vinclozolin	5	0.072	108ns
Vinclozolin	25	0.081	122ns
Vinclozolin	50	0.043	65*

†Data are mean values of four culture plates/treatment ± S.E.
[a]Number of colonies/3 × 10^2 seeded cells per plate.
[b]Values are expressed as total number of colonies per treatment relative to the negative control (solvent-treated plates).
*,**Differences against control calculated by the Student's t test on on the mean number of colonies scored per plates; *P < .05, **P < .01; ns = not significant

RESULTS

Table II reports the data from cytotoxicity assays performed with three doses of each pesticide in the absence of metabolic activation. Dose-employed in this test for each chemical were chosen according to the cytotoxicity shown in short-term tests of genotoxicity previously performed on human peripheral blood lymphocytes (personal observations). Only the highest assayed dose of each pesticide was toxic for cultured BALB/c 3T3 cells. Thus subtoxic or nontoxic doses (50 μg/ml for cyanazine, diflubenzuron and procymidone and 25 μg/ml for dithianon and vinclozolin) were chosen in order to assay cell transformation activity of pesticides.

Results from the level-I cell transformation test, obtained after treatment of cell cultures with a single dose of each pesticide in the absence of metabolic activation, are shown in Table III.

The mean number of foci/plate detected in the cultures treated with cyanazine, diflubenzuron, procymidone, and vinclozolin was significantly higher than that observed in untreated cells or 0.5% DMSO-treated-controls. By contrast, the number of foci in the dithianon-treated plates did not appear statistically different from that obtained in the

TABLE III. In Vitro BALB/c 3T3 Cell Transformation, Expressed as the Number of Level-I Transformation Foci, by a Single Dose of Five Pesticides Dissolved in 0.5% DMSO (final concentration)

Treatment	Dose (μg/ml)	Plates with foci/plates scored	Mean number of foci/ plate ± S.E.	TF ($\times 10^{-4}$)[a]
Untreated	—	6/9	0.77 ± 0.22	10.2
DMSO (0.5%)	—	5/8	0.75 ± 0.25	11.4
Cyanazine	50	7/7	2.86 ± 0.40*	69.7
Diflubenzuron	50	7/7	2.28 ± 0.42**	35.1
Dithianon	25	7/7	1.85 ± 0.50***	26.4
Procymidone	50	6/6	4.83 ± 0.87*	75.5
Vinclozolin	25	6/6	3.16 ± 0.54*	39.0

[a]Transformation frequency (TF): mean number of transformation foci per number of cells at risk determined by cytotoxicity test (CE).
*-**-***Differences against controls according to distribution-free Mann-Whitney unpaired t-test; *$P < .01$, **$P < .05$, ***$.05 < P < .1$

controls under these experimental conditions. The treatment with the different pesticides led to TF values at various extents. Thus with respect to the TF values detected in the negative controls, cyanazine and procymidone led to > sixfold higher TF values, the treatment with diflubenzuron and vinclozolin gave rise to a TF value > threefold higher, whereas the TF value from dithianon-treated plates was > twofold higher. On the whole, the TF values and the statistical differences in foci distribution seem to show that cyanazine and procymidone are the most effective in inducing cell transformation.

Table IV shows results of a second cytotoxicity test performed with three doses of pesticides on BALB/c 3T3 cells cultured in the presence and in the absence of an exogenous metabolizing system (± S-9 mix). In the absence of bioactivation, the overall picture was rather similar to that shown in Table II. Indeed, cyanazine and dithianon were the most toxic agents, whereas diflubenzuron, procymidone and vinclozolin elicited cytotoxic effects only at the highest assayed doses. The presence of S-9 mix seems to lead to a deactivation of cyanazine, whereas more confusing results have been obtained with respect to diflubenzuron and vinclozolin. The mean number of colonies detected after the treatment with diflubenzuron at the dosage of 100 μg/ml, which is the only cytotoxic dose in the absence of metabolic activation, is not statistically different from that obtained in the negative controls, suggesting the pesticide deactivation by adding S-9 mix. The relative clonal efficiency of cells treated with either diflubenzuron or vinclozolin, indeed, seems to indicate an activating effect by S9-mix at the lower doses and inactivation at the highest dose. It is difficult to draw any conclusion on the basis of these results.

The results from the level-II cell transformation assay in BALB/c 3T3 cell cultures treated with pesticides are shown in Table V. In this experiment pesticides were employed at the same dose reported in Table III either in the presence or in the absence of metabolic activation. Without exogenous activation, diflubenzuron, dithianon, procymidone, and vinclozolin induced TF values significantly higher than those obtained in negative controls. Moreover, the TF value of cyanazine-treated cultures was even similar to that induced by a 20-fold lower amount of 3-MCA used as control. However, in the presence of the metabolizing system, cyanazine and diflubenzuron were unable to transform BALB/c 3T3 cells, whereas dithianon was twofold more effective than in the absence of S-9 mix. Finally, TF value of dithianon was similar to that induced by a 10-fold lower amount of the positive control, B(a)P.

Table VI summarizes all data obtained about the cytotoxic and transforming activities exerted by the assayed pesticides.

DISCUSSION

All the chemicals tested under the experimental conditions described here induce, to various extents, cell transformation. However, their relative cell-transforming potencies (effect/mmol dose) are 1–2 orders of magnitude lower than those of the greatly potent carcinogens used as positive controls (3-MCA and B(a)P). Furthermore, the addition of S9-mix seems to affect both cytotoxicity and transforming activity of some of the assayed pesticides. Cyanazine, which is cytotoxic at all the assayed doses, and diflubenzuron, whose cytotoxicity is detectable only at the highest assayed dosage, are detoxified by S9-mix, and their transforming activity is inhibited.

It is difficult to define whether the transforming activity of the assayed pesticides can be due to initiating or promoting effects or both. Controversial, often inconclusive, existing data from short-term tests measuring genotoxic endpoints do not allow any conclusions to be drawn on the activity of these compounds as mutagenic agents. We have also tested the pesticides for their ability to induce SCEs or UDS in human peripheral blood lymphocytes. These tests have yielded negative results (P. Perocco, personal communication). However, pesticides employed at the same dosages as those utilized in these assays have been able to induce cell transformation. Unlike SCEs, UDS and other short-term tests that are suitable assays for revealing the initiating events related to carcinogenesis process, i.e., gene mutations or DNA damage, BALB/c 3T3 cell transformation model may be utilized for assaying environmental agents that could exert their carcinogenic action through nongenotoxic (promoting?) mechanisms. BALB/c 3T3 cells are considered nontumorigenic cells that exhibit the properties of postconfluence inhibition of cell division and anchorage dependence. However, they undergo spontaneous morphological transformation in response to conditions of constrained growth and acquire tumorigenic properties when injected attached to glass beads into animals [Boone,

TABLE IV. Cytotoxic Effects of Three Doses of Five Pesticides Dissolved in 0.5% DMSO (final concentration) on BALB/c 3T3 Cells Cultured In Vitro in the Absence (−S9-mix) or in the Presence (+S9-mix) of an Exogenous Metabolizing System†

		S9-mix			S9-mix		
Treatment	Dose (μg/ml)	Mean number of colonies/plate	Clonal efficiency[a]	Relative clonal efficiency (%)	Mean number of colonies/plate	Clonal efficiency[a]	Relative clonal efficiency (%)[b]
Untreated		39.5 ± 2.6	.131	126	24.0 ± 2.0	.080	90
DMSO (0.5%)		31.2 ± 2.9	.104	100	26.6 ± 2.1	.088	100
3-MCA	2.5	19.0 ± 2.8*	.063	61			
B(a)P	2.5				19.2 ± 2.0*	.064	
Cyanazine	10	19.7 ± 3.0*	.065	63	27.0 ± 1.0^{ns}	.090	101
Cyanazine	50	12.5 ± 1.0**	.041	40	24.0 ± 3.2^{ns}	.080	90
Cyanazine	100	8.5 ± 1.2**	.028	27	22.0 ± 3.2^{ns}	.073	83
Diflubenzuron	10	35.0 ± 3.7^{ns}	.116	112	21.0 ± 2.1^{ns}	.070	78
Diflubenzuron	50	27.5 ± 1.9^{ns}	.092	88	23.0 ± 3.9^{ns}	.077	86
Diflubenzuron	100	20.2 ± 0.8*	.067	65	24.0 ± 2.5^{ns}	.080	90
Dithianon	5	28.2 ± 3.5^{ns}	.094	90	22.5 ± 2.6^{ns}	.075	84
Dithianon	25	20.0 ± 0.8*	.067	64	15.7 ± 0.8**	.052	59
Dithianon	50	11.0 ± 1.3**	.037	35	11.5 ± 0.9**	.038	43
Procymidone	10	27.0 ± 1.0^{ns}	.090	87	21.3 ± 1.6^{ns}	.071	80
Procymidone	50	33.5 ± 1.5^{ns}	.112	107	25.6 ± 0.6^{ns}	.085	96
Procymidone	100	21.0 ± 3.5***	.070	67	20.0 ± 2.9^{ns}	.067	75
Vinclozolin	5	32.5 ± 1.9^{ns}	.108	104	21.6 ± 3.7^{ns}	.072	81
Vinclozolin	25	29.6 ± 0.8^{ns}	.098	95	19.6 ± 0.3*	.065	74
Vinclozolin	50	19.7 ± 2.7*	.063	63	22.6 ± 5.7^{ns}	.075	85

†Data are mean values of four culture plates/treatment ± S.E.
[a]Clonal efficiency (CE): number of colonies/3 × 10^2 cells seeded per plate.
[b]Total number of colonies per treatment relative to the negative control.
*,**,***Significant difference from control (solvent-treated plates) at the Student's t test; *P .05, **P < .01, ***.05 < P < ns not significant.

TABLE V. In Vitro BALB/c 3T3 Cell Transformation, Expressed as the Number of Level-II Transformation Foci, by a Single Dose of Five Pesticides Dissolved in 0.5% DMSO (final concentration), in the Absence (−S9-mix) or in the Presen (+S9 mix) of an Exogenous Metabolizing System

		S9-mix			+S9-mix		
Treatment	Dose (μg/ml)	Plates with foci/plates scored	Mean number of foci/plate ± S.E.	TF (× 10^{-4})[a]	Plates with foci/plates scored		TF (× 10^{-4})[a]
Untreated		3/6	0.50 ± 0.22	3.8	3/9	0.33 ± 0.16	4.1
DMSO (0.5%)		3/9	0.33 ± 0.16	3.2	2/7	0.28 ± 0.18	3.2
3-MCA	2.5	7/7	3.00 ± 0.61*	47.6			
B(a)P	2.5				9/9	2.55 ± 0.47*	39.8
Cyanazine	50	6/7	1.71 ± 0.42**	41.7	1/6	0.16 ± 0.16^{ns}	2.0
Diflubenzuron	50	6/7	1.57 ± 0.36**	17.1	2/9	0.22 ± 0.14^{ns}	2.9
Dithianon	25	8/9	1.44 ± 0.33*	21.5	5/5	2.00 ± 0.63**	38.5
Procymidone	50	7/8	2.25 ± 0.67**	20.1	8/9	1.66 ± 0.33**	19.5
Vinclozolin	25	7/8	1.87 ± 0.85**	19.1	4/8	0.87 ± 0.39^{ns}	13.4

[a]Transformation frequency (TF): mean number of transformation foci per number of cell at risk determined by cytotoxicity test (CE).
*,**Differences against controls according to distribution-free Mann-Whitney unpaired t-test; *P < .01, **P < .05; ns = not significant

1974]. It is highly probable that initiated cells already are present in BALB/c 3T3 cell population as a consequence of the acquisition of an indefinite lifespan (immortalization) [Newbold et al., 1982; Barrett et al., 1986], which involves the activation and/or deregulation of particular genes [Balmain and Brown, 1988]. Whether the assayed pesticides can be considered as agents that "promote" initiated BALB/c 3T3 cells or induce further genetic alterations is an open question that requires further investigations.

It should be emphasized that even some human carcinogens, e.g., asbestos and diethylstilbestrol, that give false negative results in other short-term tests of mutagenicity can induce in vitro cell transformation [Barrett et al., 1986]. Consequently, the positive findings reported here could be

TABLE VI. Effects of Exogenous Bioactivation on Cytotoxicity and Transformation Activity by the Assayed Pesticides

	Induced effects[a]			
	Cytotoxicity			
	−S9-mix	+S9mix		
Cyanazine	++	−	++	−
Diflubenzuron	+	−	+	−
Dithianon	++	++	+	++
Procymidone	+/−	−	++	+
Vinclozolin	+/−	−	+	+

[a]++: high activity; +: low activity; −: no activity.

relevant in assessing hazard and risk associated with the human exposure to the (tested) pesticides. Indeed, they could act in the environment as "syncarcinogenic" or "co-carcinogenic" agents interacting with other genotoxic or nongenotoxic chemicals. Results reported here show that the in vitro BALB/c 3T3 cell transformation can be validated as a suitable model for the analysis of carcinogenic potential also of many other nongenotoxic pesticides.

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Adaptation to oxidative stress: effects of vinclozolin and iprodione on the HepG2 cell line

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Abstract

It is well known that the dicarboximide fungicides, vinclozolin and iprodione, induce lipid peroxidation by means of oxygen activation in fungi, but their action on mammalian cells is not yet clear. We therefore investigated the effect of 1- and 24-h treatments with vinclozolin at concentrations of 25, 50, 100 μg/ml and iprodione at concentration of 62.5, 125, 250 μg/ml on malonaldehyde and free radical production and on reduced glutathione levels in the human HepG2 hepatoma cell line. The concentrations were chosen on the basis of neutral red cytotoxicity assays. One-hour treatment with the different concentrations of either vinclozolin or iprodione increased both malonaldehyde and free radical content, and decreased reduced glutathione levels, whereas 24-h treatment decreased malonaldehyde content and free radical production, and increased reduced glutathione concentration. These results suggest that the mammalian cells respond to the initial oxidative damage caused by the two dicarboximide fungicides by means of a characteristic adaptative phenomenon within 24 h. This hypothesis is supported by the antagonized effects caused by treatment with the two dicarboximide fungicides and buthionine sulfoximine 0.5 mM, a specific and irreversible inhibitor of reduced glutathione synthesis. The data confirm that the two dicarboximide fungicides maintain their specific action in mammalian cells, although this action is masked by adaptation.

Keywords: Vinclozolin; Iprodione; Free radical damage; Reduced glutathione; Buthionine-sulfoximine; HepG2 cells

1. Introduction

The dicarboximide fungicides (DCOFs) vinclozolin and iprodione have been introduced as agricultural fungicides over the last 20 years (Orth et al., 1992; Ronis and Badger, 1995). The cyclic

Abbreviations: BSO, buthionine sulfoximine; DCFH-DA, 2'-7'dichlorofluorescin diacetate; DCOFs, dicarboximide fungicides; γGCS, γ-glutamylcysteine synthetase; GSH, reduced glutathione; MDA, malonaldehyde; TCA, trichloroacetic acid.

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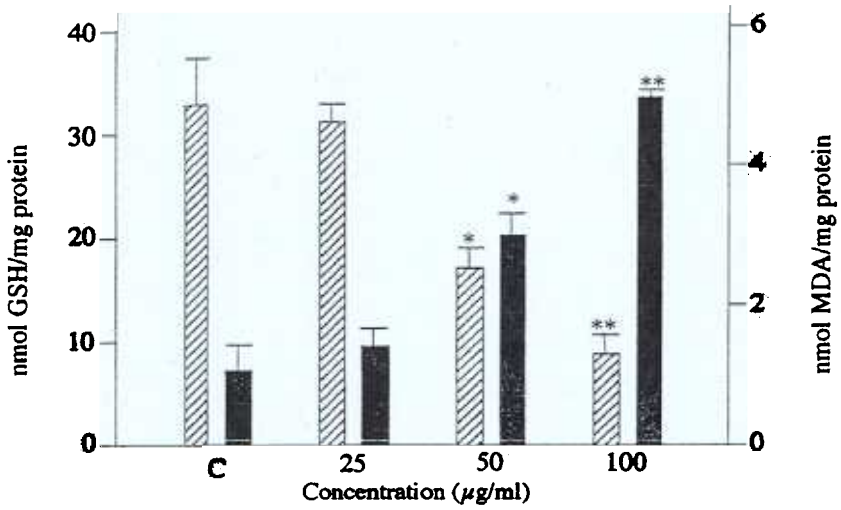

Fig. 1. Effects of different concentrations of vinclozolin on GSH (■) and MDA (■) levels in HepG2 cells treated for 1 h. The results are the mean ± S.E.M. of five experiments. C, control. * $P < 0.05$ of ** $P < 0.01$ vs control cells.

imide component in the two DCOFs may be an oxazolidine-dione (vinclozolin) or a hydantoine (iprodione), which lead to understandable differences in their degree of activity against listed pathogens and shifts in their spectrum of activity (Pommer and Lorenz, 1987).

The two DCOFs have been used for many years to protect bulb and other flower crops against Botrytis in tulips, Sclerotina bulborum in hyacinths, Botrytis curvularia and Stromatinia in gladioles (Edlich and Lyr, 1992).

The mode of action of DCOFs has been the subject of many studies, none of which have conclusively identified the primary target site because of a lack of knowledge concerning the mechanism of action of the dicarboximide group, the effects of which include mitotic instability (Georgopoulos et al., 1979), somatic chromosome segregation (Orth et al., 1992), lanosterol 14α-hydroxylase inhibition (Ronis and Badger, 1995), increased free fatty acid levels (Pappas and Fisher, 1979), and increased levels of micronucleated erythrocytes in bone marrow cells (Hrelia et al., 1996). Although these do not indicate a primary action that can be regarded as the basis for the very different effects observed in fungal cell metabolism, a current theory proposes that the active oxygen species generated by these compounds initiate lipid peroxidation (Edlich and Lyr, 1992). This induction of lipid peroxidation is probably due to oxidative damage of several cell compartments mediated by oxygen activation rather than to the direct conversion of the fungicides into free organic radicals (Edlich and Lyr, 1992).

The aims of this study were to verify whether vinclozolin and iprodione are also capable of causing O_2^- mediated radical damage in mammalian cells, and if this occurs, whether this primary lesion could be followed by an adaptative phenomenon.

To this end malonaldehyde (MDA) and reduced glutathione (GSH) contents, as well as the production of free oxygen radicals were evaluated as possible markers of damage in the HepG2 hepatoblastoma cell line (Duthie et al., 1995).

2. Materials and methods

2.1. Test materials

Penicillin-streptomycin solution, fetal bovine serum, pyruvic acid, L-glutamine, 2′-7′-dichlorofluorescin diacetate, and the materials for the GSH and MDA assays were purchased from

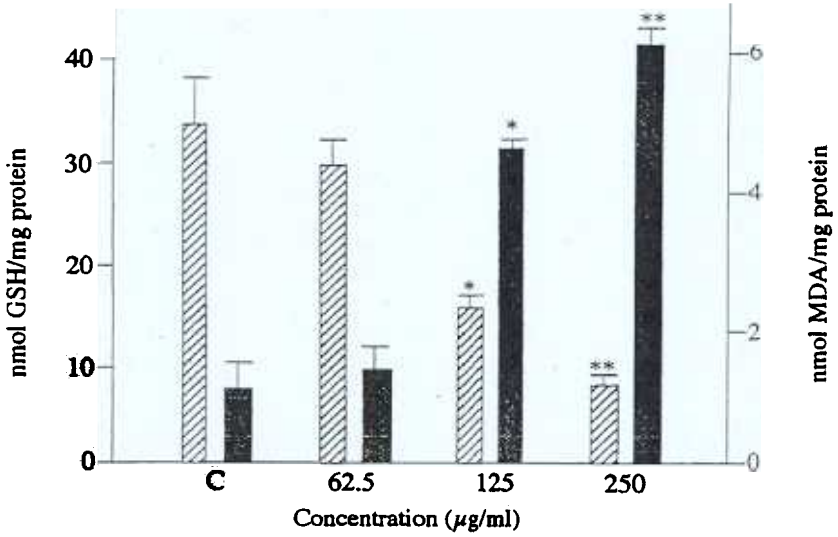

Fig. 2. Effects of different concentrations of iprodione on GSH (■) and MDA (■) levels in HepG2 cells treated for 1 h. The results are the mean ± S.E.M. of five experiments. C, control. * $P < 0.05$ of ** $P < 0.01$ vs control cells.

Sigma-Aldrich S.r.l. (Milan, Italy). RPMI 1640 medium was purchased from Labtek S.r.l. (Corsico, Milan, Italy).

Vinclozolin and iprodione were supplied by Lab Service Analytica (Bresso, Milan, Italy).

2.2. Cell cultures and treatments

The human HepG2 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 0.03% glutamine, 0.11 g/l pyruvate, 100 μg/ml streptomycin and 100 U/ml penicillin in a humidified atmosphere with 5% CO_2 at 37°C.

The cells were split 1:3 every 7 days. The medium was changed every 2–3 days and all of the enzyme activity measurements were carried out on confluent cells 7 days after subculture.

The confluent monolayers of the HepG2 cell line were treated for 1 and 24 h with a medium containing the substances at the concentrations indicated in the individual figures. Vinclozolin and iprodione were dissolved in DMSO. All of the experimental and control incubation solutions contained a 0.1% final concentration of DMSO.

2.3. Cytotoxicity assay

In order to exclude highly toxic concentrations, neutral red uptake cytotoxicity assays were performed according to the method of Zhang et al. (1990). After removing the medium, 1 ml of neutral red solution was added to each plate. After 3 h of incubation at 37°C, the solution was removed and the cells rapidly washed with 1 ml of fixative (1% formaldehyde, 1% $CaCl_2$). A 1 ml solution of 1% acetic acid and 50% ethanol was added to each plate in order to extract the dye and the plates were read at 540 nm after being left for 1 h at room temperature.

2.4. Intracellular GSH measurements

The samples for measuring GSH were processed according to the methods of Hissin and Hilf, (1976). After washing twice with phosphate buffer pH 7.4, the cultured monolayers were scraped into 6.5% TCA; 4.5 ml of phosphate EDTA buffer pH 8 was added to 0.5 ml of the 100.000 g supernatant. The final assay mixture (2 ml) contained 100 μl of the diluted supernatant, 1.8 ml of phosphate EDTA buffer pH 8, and 100 μl of the *o*-phthalaldehyde solution, containing

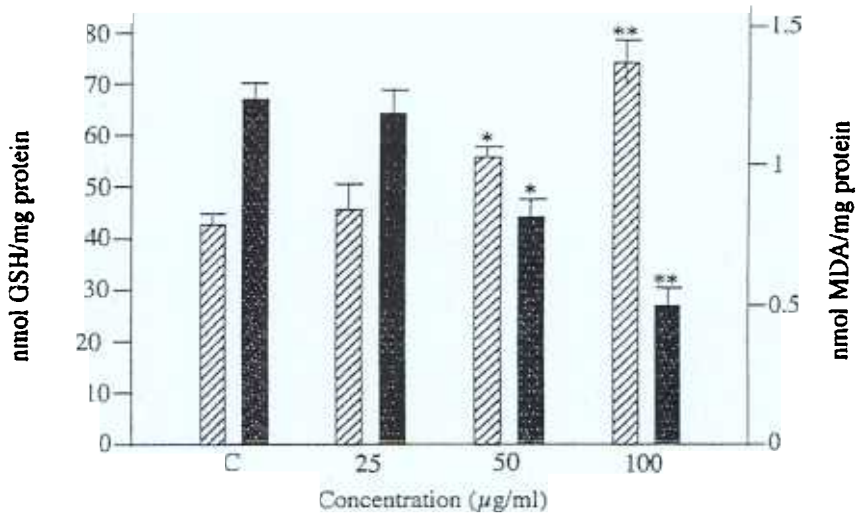

Fig. 3. Effects of different concentrations of vinclozolin on GSH (■) and MDA (■) levels in HepG2 cells treated for 24 h. The results are the mean ± S.E.M. of five experiments. C, control. * $P < 0.05$ of ** $P < 0.01$ vs control cells.

100 μg of *o*-phthalaldehyde. After thorough mixing and incubation at room temperature for 15 min the fluorescence was read at an emission wavelength of 420 nm and an excitation wavelength of 350 nm.

This assay was suitable for routine GSH measurements, and was determined to be comparable to the HPLC method in terms of its specificity and sensitivity for GSH.

Proteins were measured by the method of Lowry et al. (1951), using BSA as standard.

2.5. MDA measurements

MDA content was assessed by a modification of the thiobarbituric acid assay as previously described (Goldlin and Boelsterli, 1991).

The cells and medium were ultrasonicated for 15 s and TCA (275 μl) was added to the homogenate to give a final concentration of 5% w/v. After centrifugation (1000 × *g*, 10 min), 2 ml of the supernatant was transferred to a Pyrex tube and mixed with 2 ml of thiobarbituric acid solution (0.6% w/v). The tubes were then placed in boiling water (100°C) for 15 min. After cooling, the mixtures were extracted with 1.6 ml *n*-butanol. The butanol extracts were diluted 1:1 with *n*-butanol and fluorescence measured at 553 nm (excitation 515 nm). Tetraethoxypropane and absolute ethanol were used to prepare MDA standards.

2.6. Assay for reactive oxygen species

The production of oxygen reactive species was assessed using a method of Le Bel et al. (1990).

The cell homogenates were loaded by incubation in low Ca^{2+} PBS containing 5 μM 2′-7′-DCFH-DA for 15 min at 37°C. The 2′-7′-DCFH-DA was dissolved in ethanol as a 1000X stock solution.

The formation of the fluorescent oxidized derivative of DCFH-DA was monitored at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The cuvette holder was thermostatically maintained at 37°C.

2.7. Statistical analysis

The data are expressed as mean values ± S.E.M., and were analysed by one-way ANOVA. Statistical significance was determined by the post-hoc followed Duncan's test in which $P < 0.05$ was accepted as statistically significant.

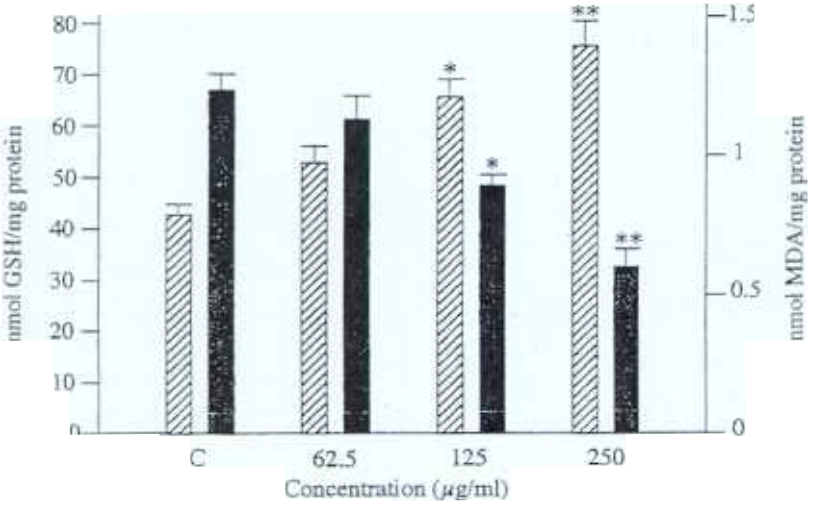

Fig. 4. Effects of different concentrations of iprodione on GSH (■) and MDA (■) levels in HepG2 cells treated for 24 h. The results are the mean ± S.E.M. of five experiments. C, control. * $P < 0.05$ of ** $P < 0.01$ vs control cells.

3. Results

The viability of the cells treated with different concentrations of vinclozolin and iprodione for 1 h, and with the same substances in the presence and absence of BSO 0.5 mM for 24 h, ranged from 100 to 75%. The tested concentrations must be considered not cytotoxic for the cell clone used. Vinclozolin 100 μg/ml and iprodione 250 μg/ml. were the maximum soluble doses under the described experimental conditions.

Treatment for 1 h with vinclozolin 50 and 100 μg/ml (Fig. 1) decreased GSH content to 17 and 9 nmol/mg protein ($P < 0.05$); following treatment with 25 μg/ml, the GSH content remained essentially unchanged. With 125 and 250 μg/ml of iprodione (Fig. 2), GSH levels decreased to 15 and 7.6 nmol/mg protein ($P < 0.05$) but did not change after treatment with 62.5 μg/ml.

On the contrary, the 24-h treatments with 50 and 100 μg/ml of vinclozolin (Fig. 3) and with 125 and 250 μg/ml of iprodione (Fig. 4) increased GSH content ($P < 0.05$) with the greater increases being observed at the highest concentrations of the two fungicides (74 and 75 nmol/mg, protein respectively).There was no statistically significant increase observed at the low concentrations of vinclozolin and iprodione, respectively.

To evaluate whether DCOFs action can be attributed to an adaptation to oxidative stress, GSH content in the presence of BSO 0.5 mM and the two DCOFs was determined. BSO was chosen because it is a specific irreversible inhibitor of γGCS (Visarius et al., 1996), which is known to be an enzyme involved in adaptation phenomena.

Fig. 5 shows the GSH content of cells treated with vinclozolin and iprodione in the presence of BSO 0.5 mM for 24 h. The γGCS inhibitor antagonized the increase in GSH content: GSH values of 46, 57 and 74 nmol/mg protein for 25, 50 and 100 μg/ml of vinclozolin, became 7.9, 7.1 and 7.6 nmol/mg protein; those of 52, 67 and 75 nmol/mg protein for 62.5, 125 and 250 μg/ml of iprodione became 7.1, 7.4 and 8 nmol/mg protein.

The two higher concentrations of DCOFs caused an increase in MDA content (Figs. 1 and 2) after 1 h treatment: the increase was to 3.1 and 5 nmol/mg protein after vinclozolin 50 and 100 μg/ml ($P < 0.05$), and to a 4.4 and 6 nmol/mg protein after 125 and 250 μg/ml iprodione ($P < 0.05$). The concentrations of 25 μg/ml and 62.5 μg/ml for vinclozolin and iprodione respectively did not modify MDA content.

On the contrary, 24 h treatment with vinclozolin (Fig. 3) and iprodione (Fig. 4) reduced

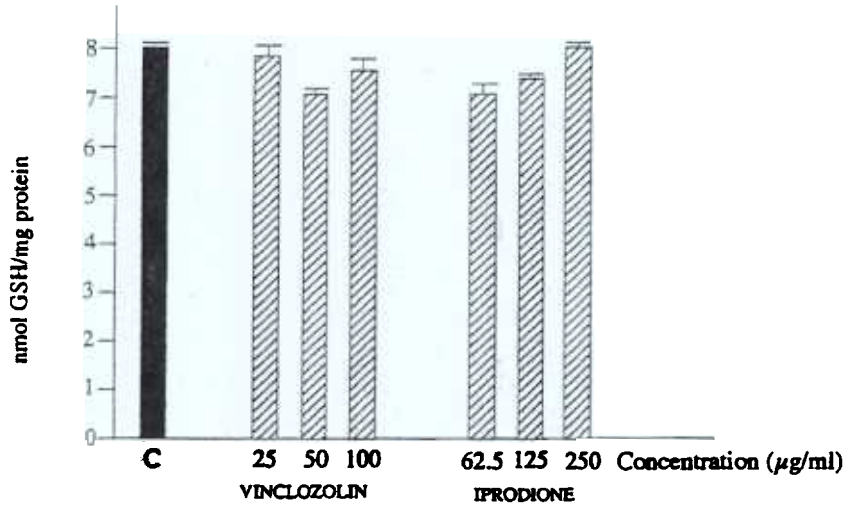

Fig. 5. Effects of different concentrations of vinclozolin and iprodione in the presence of BSO 0.5 mM on GSH levels in HepG2 cells treated for 24 h. The results are the mean ± S.E.M. of five experiments. C, control.

MDA content: treatment with 50 and 100 μg/ml of vinclozolin significantly decreased MDA content to 0.5 and 0.8 nmol/mg protein ($P < 0.05$); teatment with 125 and 250 μg/ml decreased MDA to 0.9 and 0.6 nmol/mg protein ($P < 0.05$). No significant variations were observed at the lowest concentrations of either vinclozolin and iprodione.

MDA content was also evaluated in the presence of BSO 0.5 mM. In this case, treatment with vinclozolin led to the return of MDA content to control values, although there was a trend towards an increase at the highest concentration (Fig. 6). The trend was similar but more marked after simultaneous iprodione and BSO 0.5 mM treatment (Fig. 6), with iprodione 125 and 250 μg/ml increasing MDA to 3.1 and 4 nmol/mg protein respectively ($P < 0.05$).

An increase in reactive oxygen species was observed in the cells treated for 1 h with 50 and 100 μg/ml of vinclozolin (Fig. 7) and 125 and 250 μg/ml of iprodione (Fig. 8). There was no change at the lowest concentrations of either drug, but the increases versus controls (100%) were 59 and 109% at 50 and 100 μg/ml of vinclozolin, respectively, and 64 and 111% at 125 and 250 μg/ml of iprodione. On the contrary, an opposite trend was observed in the cells treated for 24 h. As shown in Fig. 7, 50 and 100 μg/ml of vinclozolin decreased the level of reactive oxygen species to 67 and 42% of control values, 125 and 250 μg/ml of iprodione to 66 and 40% (Fig. 8). At 24 h, vinclozolin 25 μg/ml and iprodione 62.5 μg/ml did not lead to any variation in the production of reactive oxygen species.

4. Discussion

As stated in the introduction, the action of vinclozolin and iprodione involves the generation of reactive oxygen species (Pommer and Lorenz, 1987): increased levels of superoxide were found by Natvig (1982) in studies of fungal organisms, and DCOFs-treated fungi have been found to counteract this event by significantly increasing catalase activity (Edlich and Lyr, 1992). Moreover, strong evidence in favour of the above hypothesis comes from the fact that antioxidant decreases or even annihilates the oxidative stress imposed on DCOFs-treated fungal peroxidation. The addition of α-tocopherol acetate not only antagonizes the inhibition of growth, but also decreases the level of peroxides to the level of untreated controls (Fukuzawa et al., 1995).

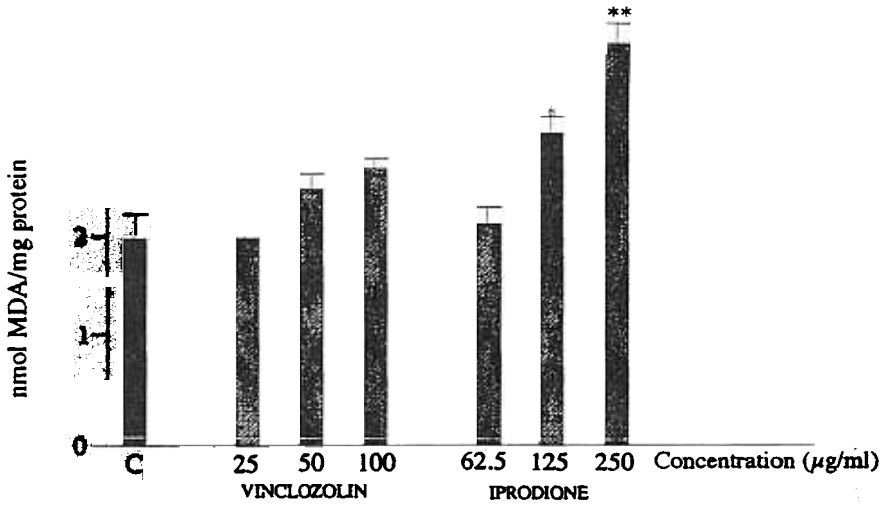

Fig. 6. Effects of different concentrations of vinclozolin and iprodione in the presence of BSO 0.5 mM on MDA levels in HepG2 cells treated for 24 h. The results are the mean ± S.E.M. of five experiments. C, control.* $P < 0.05$ of ** $P < 0.01$ vs control cells.

Our results appear to show that both vinclozolin and iprodione have a different action on mammalian cells, such as HepG2, at the two highest doses used in this study. After 24 h treatment, both DCOFs induce a decrease in MDA concentration and free radical production, together with an increase in GSH. The decrease in MDA concentration and free radical production may be a direct consequence of the increased amount of GSH, because intracellular GSH plays a central role in cell defense against oxidative stress by maintaining the intracellular reducing environment and reducing the production of reactive oxygen species (De Meyts et al., 1992; Lu et al., 1992; Tian et al., 1997). The capacity of a cell to maintain its intracellular GSH content during an oxidative challenge is therefore important to prevent the loss of cell function and integrity (Tian et al., 1997).

It is well known from the literature that the capacity of mammalian cells to maintain cell function homeostasis during oxidative stress depends on the rapid induction of protective antioxidant enzymes (Rahman et al., 1996).

The cells challenged with GSH depleting agents or sublethal oxidative stress increased GSH synthesis (Tian et al., 1997) as a consequence of an upregulation of γGCS gene expression (Rahman et al., 1996). There is evidence to suggest that the increase in γGCS activity and GSH content contributes towards greater tolerance to higher degrees of oxidative stress (Rahman et al., 1996; Tian et al., 1997).

Rahman et al. (1996) have demonstrated increased intracellular GSH levels associated with increased γGCS activity in the alveolar epithelial cells exposed for 24 h to menadione and hydrogen peroxide. The free radicals generated by menadione and hydrogen peroxide may modulate AP-1 and hARE transcription factor, and induce transcription by promoting their binding to the oligonucleotide consensus region of the γGCS-heavy subunit promoter. It has recently been demonstrated that cigarette smoke, which contains 10^{14}–10^{16} free radicals/puff, increases GSH content and γGCS heavy subunit expression in human alveolar type II cells (Rahman et al., 1996). The treatment of L2 epithelial cells with different concentrations of tert-butylhydroquinone also leads to an up-regulation of both γ-glutamyltranspeptidase and γGCS as an adaptative response to oxidative stress (Choi et al., 1997). Moreover, other quinones such as DMNQ and menadione induce GSH to a similar extent and are thus also expected to have a similar protective effect (Shi et al., 1994; Forman et al., 1995).

These data show that the increased GSH levels, as a protective response, is always preceded by an initial harmful response due to free radicals (Shi et al., 1994; Rahman et al., 1996, 1997). Woods et al. (1992) have also demonstrated that the initial effect of methyl mercury hydroxide treatment is pronounced renal GSH depletion, whereas prolonged treatment enhances kidney GSH content.

Like those data already described in the literature, our results show that the increase in GSH, and the decrease in MDA and free radicals, caused by the DCOFs after 24 h was preceded by radical effects in the short term (1 h) characterised by a drop in GSH levels and an increase in MDA and free radicals.

The trend of this phenomenon therefore suggest that it is a characteristic of cell adaptation.

The confirmation that γGCS is specifically implicated in this phenomenon is given by the fact that, if treated with BSO, a potent irreversible inhibitor of γGCS (Visarius et al., 1996), the increase in GSH content secondary to treatment with the two DCOFs is completely annulled. Moreover, the decrease in GSH levels is accompanied by an increase in MDA content. These data support the idea of the participation of γGCS mRNA excluding other possible glucogenic pathways.

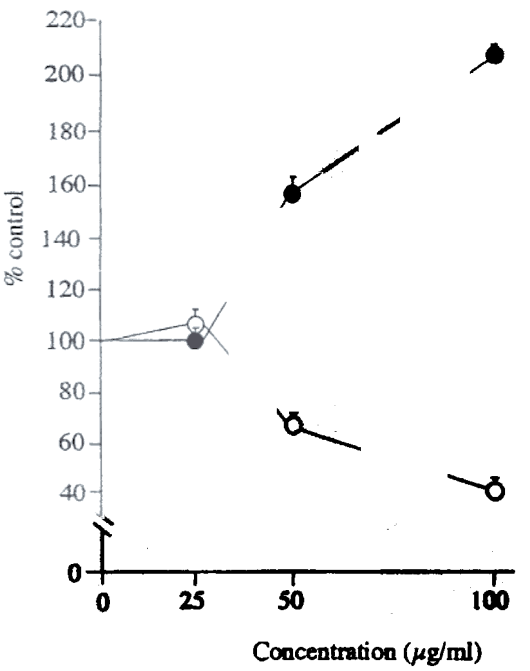

Fig. 7. The formation of reactive oxygen species at different doses of vinclozolin 1 h (●) and vinclozolin 24 h (○). The data are presented as the arithmetic mean percent of the control values.

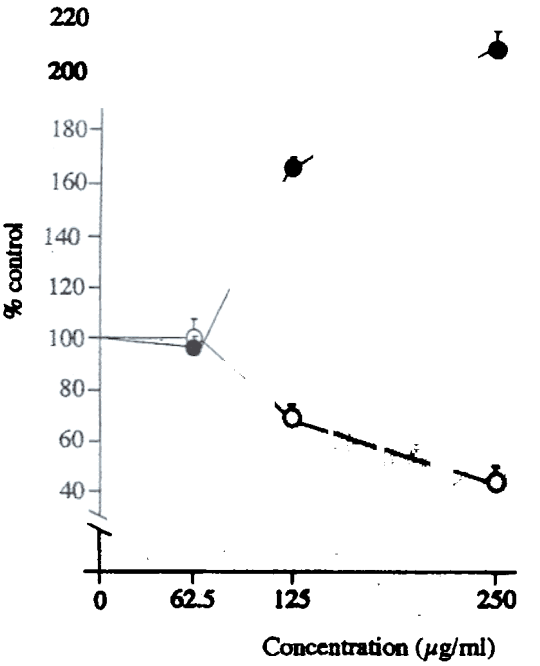

Fig. 8. The formation of reactive oxygen species at different doses of iprodione 1 h (●) and iprodione 24 h (○). The data are presented as the arithmetic mean percent of the control values.

In conclusion, we have shown that prolonged in vitro treatment with vinclozolin and iprodione leads to a progressive increase in GSH levels. This effect is probably associated with an increased in the relative abundance γGCS mRNA. These findings provide evidence that increased synthesis of GSH occurs as an adaptative response to vinclozolin and iprodione-induced oxidative stress in mammalian cells and confirm that vinclozolin and iprodione are also capable of maintaining their specific action of inducing free radical damage in mammalian cells, although this action is masked by an adaptative phenomenon.

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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

FILE COPY

JUL 27, 1994

MEMORANDUM

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

SUBJECT: Carcinogenicity Peer Review of Iprodione

FROM: Esther Rinde, Ph.D. E. Rinde
Manager, Carcinogenicity Peer Review Committee
Science Analysis Branch
Health Effects Division (7509C)

and

Linda Taylor, Ph.D. Linda Taylor 4/25/94
Toxicology Branch II, Section II
Health Effects Division (7509c

TO: Steven D. Robbins
Product Manager # 21
Fungicide-Herbicide Branch
Registration Division (7505C)

and

Kathy Davis, Section Chief
Accelerated Reregistration Branch, Review Section II
Special Review and Reregistration Division (7508W)

THROUGH: Penelope Fenner-Crisp, Ph.D.
Director, Health Effects Division (7509C)

The Health Effects Division Carcinogenicity Peer Review Committee (CPRC) met on February 23, 1994 to discuss and evaluate the weight-of-the-evidence on Iprodione with particular reference to its carcinogenic potential. The CPRC concluded that Iprodione should be classified as a Group B2 - Probable Human Carcinogen - based on evidence of tumors in both sexes of the mouse and in the male rat, and that for the purpose of risk characterization, a low dose extrapolation model be applied to the animal data for the quantification of human risk (Q_1^*). The CPRC recommended that a Q_1^* be determined for the hepatocellular combined adenoma/carcinoma for both sexes of the mouse and also separately for the testicular tumors in the male rat.

SUMMARY

Administration of Iprodione in the diet for 99 weeks to Charles River CD-1 mice resulted in statistically significant increases in hepatocellular tumors in both sexes of Charles River CD-1 mice. Female mice also had a statistically significant increase in ovarian luteomas.

Administration of Iprodione in the diet to Charles River Sprague Dawley rats for 2 years resulted in a statistically significant increase in testicular interstitial cell tumors in males. [Details are provided in Section F. "The Weight of Evidence".]

Iprodione was tested in a variety of mutagenicity studies and found to be negative in all but a Bacillus subtilis assay.

Iprodione is structurally similar to Procymidone which was classified by the CPRC as a Group B2 carcinogen, and Vinclozolin (not yet peer reviewed). Both Procymidone and Vinclozolin are associated with testicular tumors in the rat and liver tumors in the mouse (as well as other tumor types).

There were no data provided on hormonal mechanisms to attribute the carcinogenic response as being secondary to the toxicity of the chemical.

The classification of Group B2 was based on evidence of increased incidences of tumors in 2 species: hepatocellular tumors in both sexes of the mouse, ovarian tumors in female mice and testicular interstitial tumors in male rats.

A. Individuals in Attendance at the meetings:

1. Peer Review Committee: (Signatures indicate concurrence with the peer review unless otherwise stated.)

William Burnam

Karl Baetcke

Marcia Van Gemert

Elizabeth Doyle

Hugh Pettigrew

Esther Rinde

2. Reviewers: (Non-committee members responsible for data presentation; signatures indicate technical accuracy of panel report.)

Linda Taylor[1] 6/23/94

Byron Backus

Clark Swentzel 6/23/94

Lori Brunsman

Lucas Brennecke[2] 6/29/94
(PAI/Clement)

3. Other Attendees:

Karen Whitby, Bernice Fisher (HED)

[1]Also a member of the PRC for this chemical; signature indicates concurrence with the peer review unless otherwise stated.

[2]Signature indicates concurrence with pathology report.

B. Material Reviewed:

The material available for review consisted of DER's, one-liners and other data summaries prepared and/or supplied by Dr. Linda Taylor, and tables and statistical analysis by Lori Brunsman. The material reviewed is attached to the file copy of this report.

C. Background Information

Iprodione [3-(3,5-dichlorophenyl)-N-(1-methylethyl)-2,4-dioxo-1-imidazolidinecarboxamide]; [3-(3,5-dichlorophenyl)-N-isopropyl-2,4-dioxoimidazolidine-1-carboxamide] is a broad spectrum, contact fungicide formulated for use on a variety of crops, among them are fruit trees, berry fruit, vines, vegetable crops, cereals, rice, oilseed rape, sunflower, ornamental crops, and turf. It is known by the tradename Rovral® and Glycophene. The molecular formula is $C_{13}H_{13}Cl_2N_3O_3$ (MW 330.16). Iprodione has a vapor pressure of $< 10^{-6}$ mm Hg at 20° C and a solubility of 13 mg/L in water at 20° C. [See Figure 1 (file copy) for structure].

The Caswell (or Tox Chem) Number of Iprodione is 470A, the Shaughnessey Number is 109801, and the Chemical Abstracts Registry Number (CAS No.) is 36734-19-7.

D. Evaluation of Carcinogenic Evidence

1. Chambers, PR, Crook, D, Gibson, WA, Gopinath, C, and Ames, SA. Iprodione: Potential Tumorigenic and Toxic Effects in Prolonged Dietary Administration to Rats. Study # RNP 346/920808, Huntingdon Research Centre, Ltd., Department of Rodent Toxicology, England; dated December 15, 1992. MRID # 426378-01 [Document # 010570].

Experimental Design: Iprodione was administered in the diet to 60 Sprague-Dawley [Crl:CD(SD)BR] rats/sex/group [main study] for two years at dose levels of 0, 150 [♂♂ 6.1/♀♀ 8.4 mg/kg/day], 300 [♂♂ 12.4/♀♀ 16.5 mg/kg/day], or 1600 [♂♂ 69/♀♀ 95 mg/kg/day] ppm. There was a 52-week interim sacrifice of 10 additional rats/sex/group.

Non-neoplastic Lesions: At the interim sacrifice, males at the high-dose level displayed an increase in the incidence of lesions in the adrenals, and there was an increase in the incidence of centrilobular hepatocyte enlargement in males at 300 and 1600 ppm. Females at the high-dose level displayed an increase in extramedullary hemopoiesis in the spleen, an increase in centrilobular hepatocyte enlargement, and an increase in the incidence of generalized rarefaction and fine vacuolation of the zona fasciculata in the adrenals compared to the control and other dose groups.

In the rats fed the test material for 2 years, interstitial cell hyperplasia in the testes, reduced spermatozoa in the epididymides, and absent/empty secretory colloid cells or reduced secretion in the seminal vesicles were observed in males at the 300 and 1600 ppm dose levels. Atrophy of the seminiferous tubules in the testes,

with atrophy of the prostate and absence of spermatozoa in the epididymides were observed at 1600 ppm. Centrilobular hepatocyte enlargement was increased in males at the high-dose level. Adrenal lesions were observed in both sexes at the 300 and 1600 ppm dose levels, although the males displayed more lesions than the females. In the high-dose females, there was an increased incidence of tubular hyperplasia in the ovaries and increased sciatic nerve fiber degeneration compared to the controls. Hemosiderosis was increased in females at the two highest dose levels. The NOEL for non-neoplastic changes was 150 ppm [♂♂ 6.1/♀♀ 8.4 mg/kg/day] and the LEL was 300 ppm [♂♂ 12.4/♀♀ 16.5 mg/kg/day].

Neoplastic lesions: There was an increase in the incidence of both unilateral and bilateral benign interstitial cell tumors in the testes of males at the 1600 ppm dose level. There was a dose-related increasing trend and a significant difference in the pair-wise comparison of the 1600 ppm dose group with controls for testicular tumors [Table 1] which exceeds the historical control incidence [Table 2].

Table 1. Testicular Interstitial Cell Tumor Rates♦ and Peto's Prevalence Test Results (p values)

Dose	0 ppm	150 ppm	300 ppm	1600 ppm
Incidence (%)	3♦/51 (6)	7/57 (12)	7/52 (13)	29/59 (49)
p value	0.000**	0.130	0.104	0.000**

♦ # of tumor bearing rats/# of rats examined, excluding those that died or were sacrificed before observation of the first tumor.
♦ First benign tumor observed at week 74, dose 0 ppm.
Note: Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

Table 2. Historical Control Incidence of Testicular Tumors

Tumor/Study #	Incidence [# with tumor/# examined]
Interstitial Cell Tumor	
8901	2/50
8902	4/50
8903	1/50
8904	0/50
8905	0/49
8906	4/50
8907	5/50

Consideration of Adequacy of Dose Level Selection: The statistical evaluation of mortality [Brunsman memo dated 1/27/94] indicated a significant decreasing trend in mortality with increasing doses of Iprodione in male rats. Female rats showed no significant incremental changes with increasing doses of Iprodione.

Body weight gains were decreased in both sexes at the 1600 ppm dose level compared to control values during the 0-12 week interval and at other intervals also. At week 12, body-weight gain was 83.6% of the control value in the males and 80.7% of the control value in females at the highest dose level. Overall, body-weight gains were 86% and 92% of control values in the high-dose males and females, respectively.

Based on the above, the CPRC considered the doses used in this study to be adequate for assessing the carcinogenic potential of Iprodione in the rat.

In the 90-day study [MRID # 429607-01], dose levels of 0, 1000 ppm [78 ♂/89 ♀ mg/kg], 2000 ppm [151 ♂/184 ♀ mg/kg], 3000 ppm [252 ♂/266 ♀ mg/kg], and 5000 ppm [351 ♂/408 ♀ mg/kg] resulted in signs of toxicity [pilo-erection, hunched posture, pale and/or cold extremities, an emaciated appearance, decreased body weight {♂♂ 75%, 52%, and 39% of control and ♀♀ 86%, 70%, and 55% of control at 2000, 3000, and 5000 ppm, respectively}, decreased body-weight gain {♂♂ 61% (2000 ppm) and 26% (3000 ppm) of control; ♀♀ 70% (2000 ppm) and 38% (3000 ppm) of control; negative gain at 5000 ppm for both sexes}, decreased food consumption {81% (♂♂ at 2000 ppm} and 69%/79% {♂♂/♀♀ at 3000 ppm}/food efficiency] leading to the early termination [week 8] of the 5000 ppm dose groups.

In a previous chronic toxicity/carcinogenicity study in Charles River CD outbred albino rats, no treatment-related tumors were reported, although the incidence of testicular interstitial cell tumors was 2, 2, 4, and 5 out of 60 rats/group at dose levels of 0, 125, 250, and 1000 ppm, respectively.

2. Chambers, PR; Crook, D; Gibson, WA; Read, RM; and Gopinath, C. May 10, 1993. IPRODIONE Potential Tumorigenic Effects in Prolonged Dietary Administration to Mice. Study No. RNP 359/921240; dated May 10, 1993. MRID No. 428250-02 [Document # 010570].

Experimental Design: Iprodione was fed to Crl:CD®-1 (ICR) BR mice (50/sex/group-Main study) at dose levels of 0, 160 [♂♂ 23/♀♀ 27 mg/kg/day], 800 [♂♂ 115/♀♀ 138 mg/kg/day], or 4000 [♂♂ 604/♀♀ 793 mg/kg/day] ppm for at least 99 weeks [or until the 52-week interim sacrifice of 15 additional mice/sex/group].

Non-Neoplastic Lesions: **Interim Sacrifice** - In the liver, mice of both sexes displayed an increase in the incidence and degree of centrilobular hepatocyte enlargement compared to the controls, and centrilobular hepatocyte vacuolation was observed in the majority of high-dose females compared to the control incidence. These findings are consistent with the increases in liver weight and plasma GPT and GOT observed in the groups. Although the incidence and degree of fat in the hepatocytes were similar among the groups, a difference in distribution was noted; i.e., control, low- and mid-dose mice displayed fat in all zones while in the high-dose mice, fat was confined to the periportal hepatocytes.

The majority of high-dose females displayed hypertrophy of the cells of the zona fasciculata of the adrenal gland. No other group displayed this lesion. The lesion correlates with the increased adrenal weight observed in these females, but no morphological correlation was observed to account for the increased adrenal weight observed in high-dose males.

Only high-dose males displayed generalized vacuolation and hypertrophy of the interstitial cells of the testes. In females, only high-dose mice displayed luteinization of the interstitial cells of the ovary. In the cervix and vagina, epithelial thickening, usually with keratinization, was observed more frequently in the treated females compared to the controls, but no dose-response was evident.

Terminal - LIVER - At the high-dose level (both sexes), there was a significantly increased incidence of single and multiple areas of enlarged eosinophilic hepatocytes and focal fat-containing hepatocytes compared to the control values. The incidence and degree of centrilobular hepatocyte enlargement were increased significantly at the high-dose level in both sexes, and the incidence of minimal centrilobular hepatocyte enlargement was increased at the mid-dose level in females compared to the control mice. Additionally, at the high-dose level, the incidence and degree of pigmented macrophages and the degree of centrilobular hepatocyte vacuolation were increased significantly in male mice compared to the control male mice. TESTES - There was an increased incidence of generalized vacuolation/hypertrophy of the interstitial cells of the testes in the mid- and high-dose mice. OVARIES - There was a dose-related increase in female mice displaying luteinization of the interstitial cell of the ovary, but statistical significance was not attained at any dose level. The NOEL for non-neoplastic changes was 160 ppm [♂♂ 23/♀♀ 27 mg/kg/day], and the LEL was 800 ppm [♂♂ 115/♀♀ 138 mg/kg/day].

Neoplastic Lesions: **Interim** - Very few tumors were observed at the interim sacrifice. Pulmonary adenoma [benign] was observed in one low-dose female and one mid-dose female, and pulmonary adenocarcinoma [malignant] was observed in one control male and one high-dose male. One male in the mid- and one male in the high-dose groups displayed a benign liver cell tumor. One benign cystadenoma in the Harderian gland was observed in the low-dose male group.

Terminal - LIVER: At the high-dose level, there was a significant increase in the incidence of benign and malignant liver cell tumors in both sexes compared to the control. The adenomas found at the control, low-, and mid-dose levels in males were all in mice at the interim or terminal sacrifice, while 6 of the 25 adenomas in the high-dose males, 1 of 2 in the mid-dose females and 5 of 21 in the high-dose females were found in mice dying on test. In the Qualita-

tive Risk Assessment memo, analysis indicates that male mice had significant increasing trends and significant differences in the pair-wise comparisons [all at $p < 0.01$] of the 4000 ppm dose group with the controls, for liver adenomas, carcinomas, and combined adenomas and/or carcinomas [Table 3]. Female mice had significant increasing trends in liver adenomas, carcinomas, and combined adenomas and/or carcinomas also [all at $p < 0.01$], and there were significant differences in the pair-wise comparisons of the 4000 ppm dose group with the controls for liver adenomas and combined adenomas and/or carcinomas [both at $p < 0.01$]. The incidence of both benign and malignant liver tumors in females at 4000 ppm is outside the historical control data, as is the incidence of carcinoma in the high-dose males [Table 4]. It is to be noted that all male groups [including the concurrent control] displayed a higher incidence of carcinomas than observed in the historical control.

Group Dose Tumor	Table 3. Liver Tumor Rates¦ and Exact Trend Test & Fisher's Exact Test Results (p values)			
	0	160	800	4000
MALES				
adenoma (%)	4/62 (6)	4/62 (6)	8♦/62 (13)	25/63 (40)
p value	0.000**	0.641	0.182	0.000**
carcinoma (%)	4/62 (6)	3/62 (5)	6/62(10)	15♠/63 (24)
p value	0.000**	0.500♥	0.372	0.006**
combined (%)	7/62 (11)	6/62 (10)	11/62 (18)	27/63 (43)
p value	0.000**	0.500♥	0.223	0.000**
FEMALES				
adenoma (%)	1/47 (2)	1/49 (2)	2/49 (4)	21⊕/48 (44)
p value	0.000**	0.742	0.516	0.000**
carcinoma (%)	1/47 (2)	1♣/49	0/49 (0)	6/48 (12)
p value	0.003**	0.742	0.490♥	0.059
combined (%)	2/47 (4)	2/49 (4)	2/49 (4)	21/48 (44)
p value	0.000**	0.676	0.676	0.000**

¦ # of tumor bearing mice/# of mice examined, excluding those that died before week 52.
♥ Negative change from control.
♦ First ♂ adenoma observed at week 52, dose 800 ppm.
♠ First ♂ carcinoma observed at week 83, dose 4000 ppm.
⊕ First ♀ adenoma observed at week 75, dose 4000 ppm.
♣ First ♀ carcinoma observed at week 79, dose 160 ppm.
Note: Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If , then $p < 0.05$. If , then $p < 0.01$.

Table 4. Historical Control Incidence of Liver Tumors		
Tumor/Study #	# males with tumor/# examined(%)	# females with tumor/# examined(%)
Benign Tumor only		
8911	5/52 (10)	0/52
8912	7/52 (13)	1/52
8913	5/52 (10)	0/52
8914	6/52 (12)	0/52
8915	9/52 (17)	0/52
8916	11/50 (22)	0/50
8917	6/50 (12)	0/50
8918	10/56 (18)	0/56
Malignant Tumor only		
8911	1/52 (1.9)	0/52
8912	0/52 (0)	0/52
8913	1/52 (1.9)	0/52
8914	2/52 (3.8)	0/52
8915	1/52 (1.9)	0/52
8916	1/50 (2.0)	0/50
8917	1/50 (2.0)	0/50
8918	2/56 (3.6)	0/56
Any Liver Tumor		
8911	6/52 (11.5)	0/52
8912	7/52 (13.5)	1/52
8913	6/52 (11.5)	0/52
8914	8/52 (15.4)	0/52
8915	10/52 (19.2)	0/52
8916	12/50 (24)	0/50
8917	7/50 (14)	0/50
8918	12/56 (21.4)	0/56

OVARIES: The incidence of luteoma of the ovaries was increased [difference in pairwise comparison significant at $p<0.05$], and there was a significant increasing trend in ovarian luteomas [Table 5]. All tumors were found at terminal sacrifice. The incidence at the high dose is slightly greater than observed in the historical control data [Table 6].

Group Dose (ppm) Lesion	Table 5. Ovarian Tumor Rates\ and Exact Trend Test & Fisher's Exact Test Results (p values)			
	0	160	800	4000
FEMALES luteoma (%) p=	0/47 (0) 0.012*	2♦/48 (4) 0.253	1/49 (2) 0.510	5/48 (10) 0.030*

\ # of tumor bearing mice/# of mice examined, excluding those that died before week 53.
♦ First luteoma observed at week 99, dose 160 ppm.
Note: Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If , then $p < 0.05$. If , then $p < 0.01$.

Table 6. Historical Control Incidence of Luteoma	
Tumor/Study #	# with tumor/# examined (%)
Luteoma	
8911	2/51 (3.9)
8912	0/52
8913	0/52
8914	1/51 (2)
8915	1/52 (2)
8916	2/50 (4)
8917	4/50 (8)
8918	0/56

KIDNEY: Clear cell carcinoma (malignant) was observed in the kidney of one high-dose male at termination.

Consideration of the Adequacy of Dose Selection: There was no apparent effect of treatment on survival, although the high-dose group displayed the highest mortality rate for both sexes. The statistical evaluation of mortality [memo from Brunsman to Taylor dated 1/27/94] indicated no significant incremental changes with increasing dose in either sex. The most frequently occurring probable cause of death was amyloidosis, with the high-dose males and all treated female groups (dose-related) displaying a greater incidence than their respective controls (Table 7).

Table 7. Incidence of Amyloidosis in Mice Dying on Test

Sex	MALES				FEMALES			
Dose level (ppm)	0	160	800	4000	0	160	800	4000
# dying due to amyloidosis (%)	11 (48)	13 (50)	8 (44)	18 (62)	7 (26)	13 (45)	16 (55)	17 (53)

During the first 18-week interval, body-weight gains were comparable among the groups for both sexes. During the 18 to 45 week interval, the high-dose mice [both sexes] displayed a statistically significant decrease in body-weight gain compared to their respective control group [56 (♂) and 53 (♀) % of control value]. Overall, mice at the high-dose level [♂♂-86%; ♀♀-89%] displayed a lower body-weight gain compared to their respective control group.

Based on the above, the CPRC considered the doses used in this study to be adequate for assessing the carcinogenic potential of Iprodione in the mouse.

In a previous chronic toxicity/carcinogenicity study in Carworth CF-1 albino mice [Accession # 097201; Document # 001519], Iprodione was negative for carcinogenicity. The dose levels were 200, 500, and 1250 ppm, and the duration of the study was 18 months. Only one ovarian tumor [malignant] was reported [500 ppm]. Liver tumors were reported as follows [Table 8]:

Table 8. Liver Tumors		
Group/Tumor Type	Benign	Malignant
MALES		
0	0/60	2/60
200	2/59	0/59
500	0/60	4/60
1250	2/59	5/59
FEMALES		
0	0/60	0/60
200	0/60	0/60
500	1/58	2/58
1250	0/59	1/59

E. Other Relevant Toxicology Information:

1. Genotoxicity

Iprodione has been tested in several mutagenicity studies. With the exception of the Bacillus subtilis assay for DNA damage, Iprodione was negative in the (1) Ames assay; (2) CHO/HGPRT mammalian cell forward mutation assay, with and without metabolic activation; (3) in vitro chromosome aberration assay in CHO cells; (4) in vitro sister chromatid exchange assay in CHO cells; and (5) dominant lethal test in mice. Iprodione was positive in the Bacillus subtilis assay for DNA damage without metabolic activation.

2. Metabolism

^{14}C-Iprodione was absorbed readily from the gastrointestinal tract, metabolized, and excreted by rats of both sexes following single low [50 mg/kg] and high [900 mg/kg] oral doses and 14 repeated low [50 mg/kg] doses. Peak blood levels were observed at 4 and 2 hours, respectively, in low-dose males and females and at 6 hours in high-dose rats of both sexes. The elimination of ^{14}C from the blood was slower in males than females. There were both dose and sex-related differences noted in absorption: males absorbed a greater percentage of the low and repeated doses than females. Although levels of ^{14}C were found in most tissues monitored, the levels were $\leq$ 0.5% of the total amount administered. It is to be noted that the testes of the low-dose [50 mg/kg] males showed no detectable amount of ^{14}C; the high dose in the rat chronic toxicity/carcinogenicity study where testicular tumors were observed was 69 mg/kg. The primary route of elimination of ^{14}C following single and repeat low dose exposure was the urine, and the feces was the primary route following high-dose exposure. Dealkylation and cleavage of the hydantoin ring were the two primary steps in the metabolism of Iprodione. Hydroxylation of the phenyl ring and oxidation of the alkyl chain also occurred. The primary metabolites recovered from

the urine [both sexes] included a dealkylated derivative of Iprodione and 2 polar but unidentified compounds. Males produced larger amounts of a hydantoin ring-opened metabolite than females, and the urine of the females contained a higher proportion of unchanged parent compound than that of the males. Several urinary metabolites were not identified. The feces contained much larger amounts of unchanged parent compound than the urine, which the authors suggested was unabsorbed Iprodione and metabolites or hydrolyzed conjugates of absorbed material.

In another single oral administration study in rats using 50 mg/kg, no sex differences were apparent in the excretion profile, and both urinary elimination [≈37%♂/28%♀] and fecal excretion [56%♂/50%♀] are major routes of excretion. The metabolism of Iprodione was extensive and characterized by the large number of metabolites formed. In the urine, RP 36115, RP 32490, RP 36112, RP 36119, and RP 30228 were either confirmed or indicated. The feces contained a large proportion of parent compound; the major fecal metabolites were RP 36115, RP 36114, RP 32490, and RP 30228. A general metabolic pathway for Iprodione in the rat indicates that biotransformation results in hydroxylation of the aromatic ring, degradation of the isopropylcarbamoyl chain and rearrangement followed by cleavage of the hydantoin moiety. Additionally, structural isomers of Iprodione resulting from molecular rearrangement, as well as intermediates in the pathway, were detected.

3. Acute, Subchronic, and Chronic Toxicity Data

The acute oral LD_{50} for Iprodione Technical in rats was > 2.5 mg/-kg, Tox. Cat. III. An acute oral study in mice gave an LD_{50} of 4 ♂/4.4 ♀ g/kg, Tox. Cat. III. In a 5-month feeding (150, 500, 1000 ppm) study [Accession # 232702; Document # 001519] in rats, the NOEL was set at 1000 ppm [HDT]. In a 3-month dog study [Accession # 232702; Document # 001519], the NOEL was set at 2400 ppm, the LEL at 7200 ppm, based on liver hypertrophy and increased SAP [doses of 800, 2400, and 7200 ppm]. No significant systemic toxicity was observed in a 3-week dermal study [MRID # 420232-01; Document # 009575] at dose levels of 100, 500, and 1000 mg/kg. The NOEL was set at 1000 mg/kg [HDT].

Two one-year dog studies have been performed. In the first study [Accession # 255951; Document #'s 004439 and 005882], the NOEL was set at 100 ppm, the LEL at 600 ppm, based on decreased prostate weight and an increased number of erythrocytes with Heinz bodies in males. The dietary levels tested were 0, 100, 600, and 3600 ppm. In the second study [MRID # 422111-01; Document # 009548], performed as a bridging study to establish a higher no-effect level, dose levels of 0, 200, 300, 400, and 600 ppm were tested. The NOEL was

set at 400 ppm [17.5 ♂♂/18.4 ♀♀ mg/kg], the LEL at 600 ppm [24.6 ♂♂/26.4 ♀♀ mg/kg], based on depressed red blood cell parameters.

4 Structure-Activity Correlations

Iprodione is structurally similar to Procymidone and Vinclozolin.

a) **Procymidone** - [N-(3,5-dichlorophenyl)-1,2-dimethylcyclopropane-1,2-dicarboximide)], a fungicide, has been tested in a rat chronic toxicity/carcinogenicity study and a mouse carcinogenicity study. In the rat [Osborne-Mendel] study, Procymidone was associated with the appearance of tumors in both sexes. In males, there was a statistically significant increase in testicular interstitial cell adenomas at the 1000 and 2000 ppm dose levels, and a statistically significant dose-related increasing trend in these tumors, which appeared earlier than in the controls. In females, there was a statistically significant increasing trend in pituitary adenomas, as well as a significant difference in the pair-wise comparison of controls and both the 1000 and 2000 ppm dose groups for pituitary adenomas. Additionally, there was an increase in stromal hyperplasia of the ovaries at the 2000 ppm dose level, but no dose-related increase in ovarian tumors. In males, there was a dose-related increase in testicular interstitial cell hyperplasia at the 1000 and 2000 ppm dose levels. Liver cytomegaly occurred in treated rats only and it was dose-related in both sexes. In the mouse [$B_6C_3F_1$], there was a significant dose-related positive trend in hepatoblastomas [rare variant of hepatocellular carcinoma] in males, and significant dose-related positive trends in hepatocellular adenomas and in combined adenomas and/or carcinomas, with a significance in the pair-wise comparison of controls and the highest dose groups in both hepatocellular adenomas and in the combined adenomas and/or carcinomas in females. Procymidone was negative in the in vitro UDS assay, the in vitro chromosome aberration [CHO cells] assay, and the Ames assay.

b. **Vinclozolin** - [3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidin-2,4 dione] is a fungicide, bactericide, and wood preservative. Vinclozolin is being tested in both a rat chronic toxicity/carcinogenicity study and a mouse carcinogenicity study; previous studies were found unacceptable. In an interim report of the new rat [Wistar] study, testicular and ovarian masses have been observed at 1500 and 4500 ppm, liver tumors [masses] at 4500 ppm, and ophthalmic lesions have been observed at all dose levels. In a subchronic study, increased liver [both sexes], adrenal [both sexes], pituitary and testes [male], and ovary [female] weights were observed at 1000 and 4500 ppm. The interim report of the mouse [C57BL/6N] study indicated that liver carcinomas were observed at 8000 ppm [HDT] in females, a dose where excessive mortality occurred. In the subchronic study in mice, hyperplasia of

testicular Leydig cells at 1000 ppm and ovarian stromal cells at 5000 ppm was observed. Increased adrenal weight was observed in dogs in both a 6-month and 1 year studies. Vinclozolin was negative in the sister chromatid exchange assay [hamsters], the in vivo reverse mutation assay, CHO/HGPRT assay with and without metabolic activation, Ames assay with and without metabolic activation, and UDS assay in rat hepatocytes. In the mouse lymphoma (forward mutation) assay, a significant reproducible increase in mutation frequency was observed with metabolic activation at insoluble concentrations.

6. **Mechanism of Action - Hormonal Effects and Tumor Induction**

The Registrant put forth an hypothesis on the mechanism of tumor formation by Iprodione, suggesting a perturbation of sex hormone regulation. A causal relationship between a possible hormonal imbalance and tumor formation is discussed in their document entitled: IPRODIONE: Carcinogenicity in Rodents, dated June 16, 1993. In light of the negative results in various genetic toxicity tests, the Registrant states that Iprodione clearly is not a genotoxic carcinogen, and the increased incidence of tumors in both rats and mice occurred only at the MTD. Considering the non-neoplastic findings in the reproductive system of male rats and female mice and the lack of genotoxic potential, the Registrant proposes that the "nongenotoxic mechanism of carcinogenesis results from a perturbation in sex hormone regulation. Tumor formation is therefore likely to be secondary to prolonged and profound hormonal imbalance at the target organ level which only occurs when animals are exposed to high dietary levels of iprodione. Such a mechanism would be expected to occur above a threshold which would need to be exceeded to overcome the powerful normal hormonal homeostasis. Consequently, quantitative carcinogen risk assessment based on the "linearized" multistage model is inappropriate in the case of iprodione. Instead, safety factors are considered to be an appropriate and adequate method for risk assessment of threshold carcinogens." The Registrant has indicated that mechanistic studies will be conducted to demonstrate the effect of Iprodione on sex hormone regulation in order to establish a link between this hormonal perturbation and the increased incidence of tumor formation.

Since no data to support the hypothesis on hormonal mechanisms were provided, the CPRC had no basis for attributing the carcinogenic response as being secondary to the toxicity of the chemical.

F. Weight of Evidence Considerations:

The Committee considered the following facts regarding the toxicology data on Iprodione in a weight-of-the-evidence determination of carcinogenic potential.

1. **Male and female Sprague-Dawley [Crl:CD(SD)BR] rats were fed Iprodione for two years at dose levels of 0, 150 [♂♂ 6.1/♀♀ 8.4 mg/kg/day], 300 [♂♂ 12.4/♀♀ 16.5 mg/kg/day], or 1600 [♂♂ 69/♀♀ 95 mg/kg/day] ppm.**

   In male rats, there was a significant [$p<0.01$] dose-related increasing trend and a significant [$p<0.01$] difference in the pair-wise comparison of the 1600 ppm dose group with the controls for testicular interstitial cell benign tumors. The incidence of both unilateral and bilateral benign interstitial cell tumors was increased at this dose level compared to the control.

   In male rats, the increased incidence of testicular tumors noted at 1600 ppm exceeds the historical control incidence for these tumors in this strain of rat.

   In female rats, there were no significant compound-related tumors observed

   In female rats, although there was no increase in the incidence of ovarian tumors, tubular hyperplasia was increased at the 4000 ppm dose levels compared to the control incidence.

   The doses used in both sexes of the rat were considered to be adequate.

2. **Male and female Crl:CD®-1 (ICR) BR mice were fed Iprodione at dose levels of 0, 160 [♂♂ 23/♀♀ 27 mg/kg/day], 800 [♂♂ 115/♀♀ 138 mg/kg/day], or 4000 [♂♂ 604/♀♀ 793 mg/kg/day] ppm for at least 99 weeks.**

   In male mice, Iprodione was associated with significant [$p<0.01$] dose-related increasing trends in liver adenomas, carcinomas, and combined adenomas and/or carcinomas.

   In male mice, there were significant [$p<0.01$] differences in the pair-wise comparisons of the 4000 ppm dose group with the controls for liver adenomas, carcinomas, and combined adenomas and/or carcinomas.

   In male mice, the increased incidences of hepatocellular tumors noted at 4000 ppm generally exceeded the available historical control incidences for these same tumor types in

mice of this strain.

In male mice, although there was no increase in the incidence of testicular tumors in the male CD-1 mice, there was a dose-related increase in the incidence of interstitial cell hyperplasia at the 300 and 4000 ppm dose levels.

In female mice, Iprodione was associated with significant [$p<0.01$] dose-related increasing trends in liver adenomas, carcinomas, and combined adenomas and/or carcinomas.

In female mice, there were significant [$p<0.01$] differences in the pair-wise comparisons the 4000 ppm dose group with the controls for liver adenomas and combined adenomas and/or carcinomas.

In female mice, the increased incidences of hepatocellular tumors noted at 4000 ppm generally exceeded the available historical control incidences for these same tumor types in mice of this strain.

In female mice, Iprodione was associated with a significant [$p<0.05$] increasing trend in ovarian luteomas, and there was a significant [$p<0.05$] difference in the pair-wise comparison of the 4000 ppm dose group with the control for ovarian luteomas.

In female mice, the increased incidence of ovarian luteomas noted at 4000 ppm in CD-1 mice exceeds the historical control incidence for these tumors in this strain of mouse.

The doses used in both sexes of the mouse were considered adequate.

3. From submitted studies, Iprodione was not mutagenic in the Ames assay, the CHO/HGPRT mammalian cell forwarded mutation assay, with and without metabolic activation, the in vitro chromosome aberration assay in CHO cells, the in vitro sister chromatid exchange assay in CHO cells and the dominant lethal test in mice. However, Iprodione was positive in the Bacillus subtilis assay for DNA damage withiut metabolic activation.

4. Iprodione is structurally related to Vinclozolin and Procymidone. Procymidone was associated with the appearance of tumors in both sexes in the sex organs and the liver, but was negative for mutagenicity. Vinclozolin, which is currently being tested for its carcinogenic potential, has been associated with adverse effects on the sex organs and liver. With the exception of the mouse lymphoma (forward mutation) assay, Vinclozolin was negative for mutagenicity.

5. Carcinogenicity in animals -- Iprodione

After a full evaluation of all of the data and supporting information regarding animal carcinogenicity, the Committee concludes that exposure to Iprodione resulted in an increased incidence of hepatocellular malignant carcinomas in males and combined heptocellular adenomas/carcinomas in both sexes of mice, ovarian luteomas in female mice, and testicular interstitial cell tumors in male rats. Structural analogs closely related to Iprodione are also carcinogenic and induce cancer and adverse effects at the same sites (liver, ovary and testis) as Iprodione. The relevance of these data to an evlaluation of Iprodione's potential for human carcinogenicity is discussed elsewhere in this document.

G. Classification of Carcinogenic Potential:

The Peer Review Committee considered the criteria contained in the EPA's "Guidelines for Carcinogen Risk Assessment" [FR51: 33992-34003, 1986] for classifying the weight of evidence for carcinogenicity.

The Peer Review Committee agreed that Iprodione should be classified as a Group B2 - probable human carcinogen and that a low-dose extrapolation methodology (Q*) be applied to the animal data. This decision was based on evidence of increased incidences of tumors in 2 species: hepatocellular tumors in both sexes of the mouse, ovarian tumors in female mice and testicular interstitial tumors in male rats. Iprodione was tested in a variety of mutagenicity studies and found to be negative in all but a Bacillus subtilis assay. Iprodione is structurally similar to Procymidone which was classified by the CPRC as a B2 carcinogen, and Vinclozolin (not yet peer reviewed). Both Procymidone and Vinclozolin are associated with testicular tumors in the rat and liver tumors in the mouse (as well as other tumor types). There were no data provided on hormonal mechanisms to attribute the carcinogenic response as being secondary to the toxicity of the chemical.

FILE

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

APR 1991

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

Subject: Peer Review of Procymidone: consideration of Science Advisory Panel recommendations

From: James N. Rowe, Ph.D. James N. Rowe 2/5/91
Review Section III
Toxicology Branch II
Health Effects Division (H7509C)

To: Ms. Susan Lewis
Product Manager, Team 21
Fungicide-Herbicide Branch
Registration Division (H7505C)

The FIFRA SAP met on November 30, 1990 to consider a set of scientific issues for Procymidone including an assessment of the weight of evidence for the carcinogenic potential of Procymidone. The SAP cited only the testicular tumors seen in the chronic rat study as support for a Group C, Possible Human Carcinogen. The Peer Review Committee reconvened on January 30, 1991 to discuss the SAP position and carcinogenicity classification. The Peer Review Committee did not concur with the SAP but generally concluded that the evidence supported a Group B_2 classification--Probable Human Carcinogen. A quantification of risk is again recommended for the testicular tumors in male rats and for the liver tumors in female mice.

Individuals in Attendance:

1. Peer Review Committee: (Signatures indicate concurrence with the peer review unless otherwise stated).

William L. Burnam

Reto Engler

Karl Baetcke

Robert Beliles

Marion Copley

Marcia Van Gemert

Kerry Dearfield

Hugh Pettigrew

Jean Parker

Esther Rinde

William Sette

Yin-Tak Woo

2. Reviewers: (Non-committee members responsible for data presentation; signatures indicate technical accuracy of panel report).

K. Clark Swentzel

Linda L. Taylor

James N. Rowe

Bernice Fisher

3. Peer Review Members in Absentia: (Committee members who were unable to attend the discussion; signatures indicate concurrence with the overall conclusions of the Committee.)

Penelope A. Fenner-Crisp

Richard Hill

Julie Du

George Ghali

4. Other Attendees:

Susan Lewis, Registration Division.

B. Material Reviewed:

The findings of the Federal Insecticide, Fungicide, and Rodenticide Act Scientific Advisory Panel conducted on November 30, 1990 were reviewed. In addition, the findings of the first Peer Review document (October 31, 1990) were also discussed.

C. Discussion of Carcinogenicity Evidence for Procymidone:

Rat carcinogenicity study

The SAP concluded that the female rat pituitary adenomas were equivocal, but concluded that the testicular tumors in the rat were evidence of a carcinogenic response in this species. The Peer Review Committee discussed the issue of pituitary tumors in females and noted that the tumors were statistically significant by both the pair-wise comparison and the trend test. It was unclear to the Committee why Osborne-Mendel rats were selected by the registrant for the study. It was also unclear where the SAP obtained their historical control range for this tumor in Osborne-Mendel rats. There were no historical data from the testing laboratory. It was further noted that the recommendation by the SAP to further evaluate the pituitary adenoma sections would not clarify concerns for historical control data specific to the laboratory in question. The Peer Review Committee felt that, at best, this tumor type was supportive to the overall assessment of carcinogenicity and did not add a great deal to the weight of evidence.

With regard to the male testicular tumors, it was noted that this strain of rat has a low historical control incidence (i.e., from the NTP program, the mean incidence was 0.4%; Goodman et al., Toxicol Appl Pharmacol, 55, 433-447, 1980) further supporting the biological significance of the tumorigenic response. All the tumor types noted in the rat are supportive of a possible hormonally-mediated effect, but it was reiterated that no definitive data were submitted to support such a mechanism.

2. Mouse carcinogenicity study

The SAP stated that the incidences of hepatic tumors in both sexes were within the range of variability expected for this species. They concluded that the evidence for hepatic carcinogenicity was absent in males and equivocal in female mice. They further stated that higher doses could have been tolerated.

With regard to female liver adenomas, there was a significant trend ($p<0.01$) and a statistically significant increase at the highest dose tested, 1000 ppm ($p<0.05$). It was unclear to the Peer Review Committee as to the source of the historical control range the SAP used for their conclusions. Based upon the historical control data provided by the test facility, the female liver adenoma incidence was outside the range. The Committee agreed that

this appeared to be a biological effect due to the chemical and that testing at higher doses would only likely confirm the original findings in the female mice.

The SAP also concluded that the hepatoblastomas noted in the male mice were not very significant and treated the blastomas as a variant of hepatic carcinomas. It was discussed within the Committee that these tumors seem to be reported more frequently of recent times. The Committee noted that the hepatoblastomas may indicate a progression to a more anaplastic state and therefore indicate an increase in the severity of the response noted at the mid-high and high dose levels. It was again noted that the mice were not administered Procymidone at high enough doses for a completely adequate assessment of carcinogenic potential. Higher dosing could result in a higher incidence of hepatic carcinomas (including hepatoblastomas).

In the first Peer Review report, only NTP Technical Report # 189 was discussed for the Procymidone metabolite analog p-chloroaniline. During this subsequent meeting it was also noted from NTP report # 351 that p-chloroaniline, a structurally-related analog of the 3, 5-dichloroaniline metabolite of Procymidone, gives good evidence that the liver is a target for tumors in mice. This supports the liver findings associated with Procymidone.

D. <u>Classification of Carcinogenic Potential</u>:

It was the general consensus of the Peer Review Committee to classify Procymidone as a Group B_2 - Probable Human Carcinogen.

The B_2 classification was based on the statistically significant increasing trend and pair-wise increase in interstitial cell adenomas in male rats, pituitary adenomas in female rats, and liver adenomas and combined adenomas/carcinomas in female mice. Although the incidences of increased tumors in both sexes of rat and female mice were benign, the Committee ascribed biological significance to these increases. Additionally, a variant (its rarity uncertain) of hepatocellular carcinoma, hepatoblastoma, had a significantly increasing trend in male mice. This may be indicative of a progression to a more anaplastic state. While the tumor incidences in mice were not greatly elevated, it was reiterated by the Committee that the effects were seen at doses well below an adequate top dose. The available evidence for a mechanism involving altered hormonal influences was not conclusive (it was noted that there was a lack of genotoxic activity by Procymidone based on available evidence). An analog of a Procymidone metabolite (p-chloroaniline) has been reported to produce liver tumors in mice, thus supporting the liver as a target organ in mice for Procymidone.

10-3-4C

FILE C 008491

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

MEMORANDUM

OFFICE OF PESTICIDES AND TOXIC SUBSTA

SUBJECT: Peer Review of Procymidone

FROM: Kerry L. Dearfield, Ph.D. Kerry Dearfield 10.26.90
Executive Secretary, Peer Review Committee
Science Analysis and Coordination Branch
Health Effects Division (H7509C)

and

K. Clark Swentzel K. Clark Swentzel 10/26/90
Section II Head, Toxicology Branch II
Health Effects Division (H7509C)

TO: Susan Lewis
Product Manager #21
Fungicide-Herbicide Branch
Registration Division (H7505C)

The Health Effects Division Peer Review Committee met on October 10, 1990 to discuss and evaluate the weight-of-the-evidence on Procymidone with particular reference to its carcinogenic potential. The Peer Review Committee voted nine votes to classify Procymidone as a Group B_2 - Probable Human Carcinogen and eight votes to classify Procymidone as a Group C - Possible Human Carcinogen. This was based on the appearance of interstitial cell adenomas in male rats, pituitary adenomas in female rats, and liver adenomas and combined adenomas/carcinomas in female mice. Additionally, a rare variant of hepatocellular carcinoma, hepatoblastoma, had a significantly increasing trend in male mice. For the purpose of risk characterization, a low dose extrapolation model applied to the experimental animal tumor data was overwhelmingly recommended for quantification of human risk (Q_1^*). A quantification of risk is recommended for the testicular tumors in male rats and for the liver tumors in female mice.

A. Individuals in Attendance:

1. Peer Review Committee: (Signatures indicate concurrence with the peer review unless otherwise stated.)

Penelope A. Fenner-Crisp Penelope A. Fenner-Crisp

For the purpose of risk characterization, a low dose extrapolation model applied to the experimental animal tumor data was overwhelmingly recommended for quantification of human risk (Q_1*). A quantification of risk is again recommended for the testicular tumors in male rats and for the liver tumors in female mice.

William L. Burnam

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John Quest

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2. Reviewers: (Non-committee members responsible for data presentation; signatures indicate technical accuracy of panel report.)

K. Clark Swentzel

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3. Peer Review Members in Absentia: (Committee members who were unable to attend the discussion; signatures indicate concurrence with the overall conclusions of the Committee.)

Esther Rinde

Marion Copley

William Sette

George Ghali

Robert Beliles

Julie Du

Yin-Tak Woo

Jean Parker

4. Other Attendees:

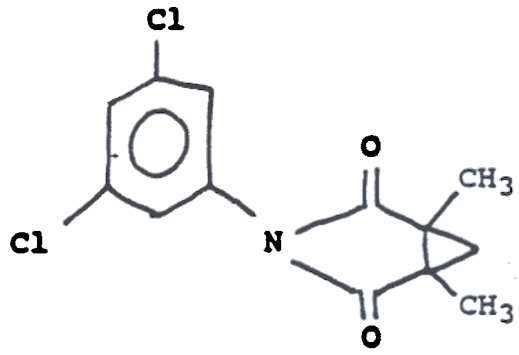
R. Bruce Jaeger, Health Effects Division
James Stone, Registration Division

B. Material Reviewed: The material available for review consisted of Data Evaluation Records (DERs) for a rat chronic toxicity/carcinogenicity feeding study and a mouse carcinogenicity feeding study. There were also summaries of a 6-month dog feeding study, rat and rabbit developmental toxicity studies, a multigeneration rat reproduction study, mutagenicity and subchronic studies. The material for presentation was prepared by K. Clark Swentzel. A qualitative risk assessment and statistical analysis for the rat and mouse carcinogenicity studies were prepared by Bernice Fisher. The material reviewed is attached to the file copy of this report.

C. Background Information: Procymidone (N-(3,5-dichlorophenyl)-1,2-dimethylcyclopropane-1,2-dicarboximide) is a fungicide that is used in Europe and other nations to control the grape disease, botrytis. It is registered for use on grapes and other fruit and vegetables in 26 countries, but not in the U.S. There are no import tolerances. In February, 1990, residues of Procymidone were discovered in two shipments of wine from Italy. In April, 1990, EPA was petitioned to establish an interim tolerance of 7 ppm for one year and then a permanent tolerance of 5 ppm for Procymidone in wine grapes. The current manufacturer is Sumitomo Chemical Co., Ltd.

The Chemical Abstracts Service (CAS) Registry number for Procymidone is 32809-16-8 and the Tox Chem Number (or Caswell number) is 704J.

Structure of Procymidone:



D. Evaluation of Carcinogenicity Evidence for Procymidone:

1. Rat Carcinogenicity Study

Reference: Keller, JG, Fitzgerald, J., Sibinovic, F., Loeb, WF, Cardy, RH, Clinton, J., and Wolfe, GW. Oral Chronic Toxicity and Oncogenicity Study in Rats, TB0100 Sumislex. Final Report No. BT-61-0112, Litton Bionetics Project No. 22048-03/13, dated June, 1986. Submitted by Sumitomo Chemical Co., Ltd. MRID #414777-03.

a. Experimental Design

Procymidone (technical, 99.8% pure) was fed to Osborne-Mendel rats (50/sex/group) in both the main and satellite groups at dose levels of 0, 100, 300, 1000, and 2000 ppm for 104 weeks. The satellite-group animals were used for clinical pathology, necropsy, and organ weight determinations (weeks 26, 52, and 78 weeks as well as terminal sacrifice).

b. Discussion of Tumor Data

Procymidone was associated with the appearance of tumors in both sexes of exposed rats (Table 1). In males, there was a statistically significant increase in testicular interstitial cell adenomas at the 1000 and 2000 ppm dose levels. These tumors tended to appear earlier than in the control and lower dose groups (although this cannot be substantiated since only one tumor occurred in each of the latter two groups). The first tumor appeared at week 77 in the 2000 ppm group. In addition, there was a statistically significant increasing dose-related trend in the appearance of the interstitial cell tumors.

In female rats, there was a statistically significant increasing dose-related trend in pituitary adenomas as well as a significant difference in the pair-wise comparison of controls and both the 1000 and 2000 ppm dose groups in the pituitary adenoma rates. The first adenoma appeared at week 52 in the 300 ppm dose group. Since female rats had a significant negative mortality trend with incremental doses of Procymidone, the Peto Prevalence test was used for the statistical analysis of the tumors, both for trends and the pair-wise comparison of controls and each dose group.

It is noted that the historical control data for the rat strain used (Osborne-Mendel) are not available from the testing facility.

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Table 1. Procymidone, Osborne-Mendel rats

Male testicular interstitial cell tumor rates[+] and Cochran-Armitage trend test and Fisher's exact test results (p values).
Female pituitary adenoma tumor rates[+] and Peto's prevalence test results (p values).

	Dose (ppm)				
	0	100	300	1000	2000
MALES					
testicular interstitial cell tumor	1/76	1/72	2/76	10/75	24/75[a]
(%)	(1)	(1)	(3)	(13)	(32)
p=	0.000**	0.738	0.500	0.004**	0.000**
FEMALES					
pituitary adenoma	21/74	21/71	24/76[b]	32/74	36/77
(%)	(28)	(30)	(32)	(43)	(47)
p=	0.00**	0.58	0.45	0.04*	0.02*

[+] Number of tumor bearing animals/number of animals examined, excluding those that died before 52 weeks (males) or those that died before observation of first tumor (females).

[a] First interstitial cell tumor observed at week 77.
[b] First pituitary adenoma observed at week 52.

NOTE: Significance of trend denoted at Control.
Significance of pair-wise comparison with control denoted at Dose Level.
If * then $p < 0.05$ and if ** then $p < 0.01$

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c. Non-Neoplastic Lesions

There was a dose-related increase in testicular interstitial cell hyperplasia in males at 1000 and 2000 ppm. Numbers of animals with testicular interstitial cell hyperplasia were 2, 0, 1, 10(1) and 13(6) for 0, 100, 300, 1000 and 2000 ppm dose groups, respectively (number in parenthesis indicates number that also had a tumor).

There were consistent, dose-related increases in liver weight throughout the study (not always statistically significant) in both

sexes. Liver cytomegaly was observed only in treated animals, and the incidence was dose-related in both sexes. For example, in males the liver/centrilobular cytomegaly incidence was 0/49, 0/48, 0/49, 11/49 and 17/46 for 0, 100, 300, 1000 and 2000 ppm dose groups, respectively. For females in the same dose groups, the incidence was 0/49, 0/50, 2/49, 25/49 and 36/50, respectively. However, no increased number of liver tumors were observed in treated animals.

There was an increase in stromal hyperplasia of the ovaries at the 2000 ppm dose level (5/50 versus 0/49 at control), but there was no dose-related increase in ovarian tumors. Only the three highest dose groups displayed some tumors. There was a slight increase in cystic hyperplasia of the uterus (at 2000 ppm dose group the incidence was 10/50 versus 4/49 at control).

The majority of the lesions were identified at terminal sacrifice in both the main and satellite groups. The satellite animals consisted of 10/sex that were scheduled for sacrifice and those that were unscheduled deaths. The above numbers for non-neoplastic lesions did not include satellite group numbers although they showed the same trends for non-neoplastic lesions as the main study groups.

d. Considerations of Adequate Dosing for Assessment of Carcinogenic Potential

Food and water consumption, clinical observations, blood and urine analyses, ophthalmoscopic evaluations and gross pathology were comparable between control and treated groups. The statistical evaluation of survival indicated that male rats had no significant mortality differences with incremental doses of Procymidone. Females had a significantly decreasing mortality trend with incremental doses of Procymidone. The female rats also had a significant decrease in mortality in the pair-wise comparison of controls and the highest dose group.

Although there were no formal statistical analyses of body-weight gain, dose-dependent decreases in body-weight gain were apparent for both sexes. At the top dose of 2000 ppm, the differences (% of control) observed at 103 weeks were 87% for males and 79% for females.

Based on the evidence presented, particularly the body-weight gain decrements and the hyperplasia in the testicular tissue where tumors were observed, the Peer Review Committee agreed that the dosing was adequate in this study for assessment of carcinogenic potential.

2. Mouse Carcinogenicity Study

Reference: Filler, R., Maloney, D., Alsaker, R., Clinton, J., Parker, G., Thakur, A., Hohing, L., and Vanatta, P. Oral Chronic Toxicity and Oncogenicity Study in Mice/Sumilex. Unpublished Report No. BT-81-0126, BT-81-0127 from Hazleton Laboratories America Inc. (1988). Hazleton Laboratories America study No. 22254. Submitted by Sumitomo Chemical Co., Ltd. MRID #414777-05.

a. Experimental Design

Procymidone (purity 99.86%) was fed to B6C3F1 mice at dose levels of 0, 30, 100, 300 and 1000 ppm to groups of 60 animals/sex/dose group. The animals were observed for 24 months, with an interim sacrifice at 52 weeks of 10 animals/sex/dose group.

b. Discussion of Tumor Data

Procymidone was associated with the appearance of liver tumors in both sexes of exposed mice (Tables 2 and 3). In male mice, there was a significant dose-related positive trend in hepatoblastomas (a rare variant of hepatocellular carcinoma). In females, there were significant dose-related positive trends in hepatocellular adenomas and in combined liver (adenomas and/or carcinomas) tumors. Female mice also had a significant difference in the pair-wise comparison of controls and the highest dose group in both hepatocellular adenomas and in the combined liver (adenoma and/or carcinoma) tumors. At the 52-week sacrifice, a hepatocellular adenoma was reported in one control male and one low-dose male.

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Table 2. Procymidone, B6C3F1 mice. Male hepatocellular tumor (adenomas, carcinomas and blastomas) rates[+] and Cochran-Armitage trend test (except where noted) and Fisher's exact test results (p values).

	Dose (ppm)				
Tumors	0	30	100	300	1000
Hepatocellular adenoma	6/60	12/60[a]	11/58	9/59	5/56
(%)	(10)	(20)	(19)	(15)	(9)
p=	0.105	0.100	0.130	0.2 9	0.549
Hepatocellular carcinoma	5/60	6/60	9/58[b]	5/59	6/56
(%)	(8)	(10)	(16)	(8)	(11)
p=	0.469	0.500	0.179	0.618	0.451
Hepatoblastoma	1/60	0/60	0/58	2/59	5/56[c]
(%)	(2)	(0)	(0)	(3)	(9)
p[++]=	0.018*	0.500	0.508	0.494	0.088
Combined carcinoma and blastoma	6/60	6/60	9/58	7/59	11/56
(%)	(10)	(10)	(16)	(11)	(20)
p=	0.055	0.619	0.267	0.487	0.114
Combined all liver tumors	12/60	18/60	20/58	16/59	16/56
(%)	(20)	(30)	(34)	(27)	(29)
p=	0.408	0.146	0.059	0.242	0.195

[+] Number of tumor bearing animals/number of animals examined, excluding those that died before 52 weeks.

[++] Exact trend test and Fisher's exact test results

[a] First adenoma observed at week 52, 30 ppm dose.
[b] First carcinoma observed at week 62, 100 ppm dose.
[c] First blastoma observed at week 105, 1000 ppm dose

NOTE: Significance of trend denoted at Control.
Significance of pair-wise comparison with control denoted at Dose Level.
If * then $p < 0.05$ and if ** then $p < 0.01$

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Table 3. Procymidone, B6C3F1 mice. Female hepatocellular tumor (adenomas, carcinomas) rates[+] and Cochran-Armitage trend test and Fisher's exact test results (p values).

	Dose (ppm)				
Tumors	0	30	100	300	1000
Hepatocellular adenoma	1/57	1/56	0/56	2/60	7/60[a]
(%)	(2)	(2)	(0)	(3)	(12)
p=	0.000**	0.748	0.504	0.519	0.036*
Hepatocellular carcinoma	1/57	1/56	2/56	4/60[b]	2/60
(%)	(2)	(2)	(4)	(7)	(3)
p=	0.342	0.748	0.493	0.198	0.519
Combined tumors	2/57	2/56	2/56	6/60	9/60
(%)	(4)	(4)	(4)	(10)	(15)
p=	0.002**	0.684	0.684	0.153	0.032*

[+] Number of tumor bearing animals/number of animals examined, excluding those that died before 52 weeks.

[a] First adenoma observed at week 87, 1000 ppm dose.
[b] First carcinoma observed at week 104, 300 ppm dose.

NOTE: Significance of trend denoted at Control.
Significance of pair-wise comparison with control denoted at Dose Level.
If * then $p < 0.05$ and if ** then $p < 0.01$

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HISTORICAL CONTROL DATA

Historical control data from the testing facility at which this study was performed are presented below. There were 9 studies in which terminal necropsies were performed during 1979 to 1987. With the exception of one study, control mice were housed for 104 weeks (exception, study O lasted 78 weeks). In these data, the rates may not contain appropriate denominators, i.e. animals at risk. Also, animals with adenomas may also include those that also had carcinomas, which is unlike the tabulations in the tables above. The range of liver tumors observed for mice are in Table 4.

Table 4. Historical Control Data, mouse studies

MALES

Study	Adenomas Rate	%	Study	Carcinomas Rate	%	Study	Blastomas Rate	%
C	0/50	0	O	1/51	2	O	0/51	0
A	1/50	2	H	3/49	6.1	A	0/50	0
O	4/51	7.8	concur.	5/60	8.3	C	0/50	0
E	4/50	8	G	6/50	12	E	0/50	0
concur.	6/60	10	D	6/49	12.2	G	0/50	0
B	5/49	10.2	C	7/50	14	B	0/49	0
D	5/49	10.2	B	11/49	22.4	D	0/49	0
F	7/49	14.3	F	11/49	22.4	F	0/49	0
H	8/49	16.3	A	16/50	32	H	0/49	0
G	12/50	24	E	19/50	38	concur.	1/60	1.7

FEMALES

Study	Adenomas Rate	%	Study	Carcinomas Rate	%
B	0/50	0	B	0/50	0
C	0/50	0	F	0/50	0
O	0/49	0	H	0/50	0
concur.	1/57	1.8	O	0/49	0
F	1/50	2	G	0/48	0
A	2/50	4	concur.	1/57	1.8
D	2/50	4	C	1/50	2
E	2/50	4	D	2/50	4
G	3/48	6.2	E	2/50	4
H	4/50	8	A	5/50	10

TUMOR TYPE	MALES	FEMALES
Hepatocellular adenoma	0-24	0-8
Hepatocellular carcinoma	6.1-38	0-10
Hepatoblastoma	0-1.7	none reported
Combined adenoma and carcinoma*	10-46	0-14

*assumes no double counting

The values in this tumor type table does not use study O.

HEPATOCELLULAR ADENOMA

With regard to adenomas, females at the 300 and 1000 ppm levels displayed incidences of 3 and 12% vs concurrent control of 2%. The top dose rate was outside the historical control range.

HEPATOCELLULAR CARCINOMA

Increased incidences of carcinomas in treated males at 100 and 1000 ppm were within the reported historical control range, and the increases were not statistically significantly greater than the concurrent control. The same is true for treated females at the top three doses.

HEPATOBLASTOMA

The increased incidence of hepatoblastoma in the 300 and 1000 ppm males was not statistically significantly greater than that observed in the concurrent controls, but was greater than the historical control range. It was noted that outside of the concurrent control, this tumor type was not found in the historical control data for males. No hepatocellular blastomas were observed in either the historical control females or the females in the current study.

COMBINED ADENOMA/CARCINOMA (Females only)

The combined incidence at the 1000 ppm dose levels (15%) was slightly outside the historical control range (0-14%), and the increase above the concurrent controls was statistically significant.

COMBINED ADENOMA/CARCINOMA/BLASTOMA (Males only)

The concurrent controls displayed an incidence of 20%, while the 100 ppm dose level showed the highest incidence (34%), which is within the historical control range (10-46%); none of the increases for combined tumor incidence was statistically significant.

c. Non-Neoplastic Lesions

There were consistent, dose-related increases in mean liver weight and relative liver weight at 105 weeks in both sexes. Liver cytomegaly was observed at the highest dose in both sexes, although the males were affected more (e.g., at 1000 ppm, the incidence of centrilobular cytomegaly in males was 43/49 versus 0/50 for the control; the incidence for females was 5/50 versus 0/50 for the control). Additionally, there was an increase in multifocal fatty changes in the high-dose males and an increase in diffuse fatty changes in the high-dose females. Dose-related cytologic alterations in the centrilobular region were reported in males at the 52-week interim sacrifice also; however, the exact nature of these alterations was not stated.

d. Considerations of Adequate Dosing for Assessment of Carcinogenic Potential

For both male and female mice, there were no statistically significant mortality differences with dose increments of Procymidone. There were no significant compound- or dose-related effects noted in body weight or body-weight gain, food or water consumption, ophthalmoscopic changes, hematology or clinical chemistry in either sex. None of this information indicated that an adequate top dose was attained in this study.

It appears from the evidence that the liver is a target organ after Procymidone administration in mice. The Peer Review Committee discussed the liver effects seen in the carcinogenicity study and the subchronic studies (see section E.3. below) in relation to adequate dosing for carcinogenic potential. Although there was some liver toxicity apparent in the subchronic studies, for example, coagulative necrosis, there was not a good dose-relationship for females even at doses up to 10,000 ppm. There was no effect on body weight gain at 2500 and 10,000 ppm. The Committee felt that while there was cytomegaly in the carcinogenicity study at the top dose, the systemic toxicity was not impressive and that dosing could have been easily higher. For males, the Committee conjectured that the significant trend in hepatoblastomas may have continued and even reached a significant pair-wise comparison. For females, the use of higher dosing may have resulted in more tumors related to dose increments, but the Committee discussed that the risk estimate itself would not likely be altered very much.

Based on these considerations, the Committee felt that this mouse carcinogenicity assay would be adequate for risk assessment although adequate top dosing may not have been attained. Furthermore, it was not considered necessary to repeat this study.

E. Additional Toxicology Data on:

1. Metabolism

Single oral doses of Procymidone (100 mg/kg) were administered to rats and mice and were found to be readily absorbed from the gastrointestinal tract and distributed to all tissues examined (MRID #414777-10). The metabolic profiles were comparable between the species. A total of ' metabolites were isolated from the urine and feces; most appear to be the result of oxidation of Procymidone by the mixed function oxidase system. Extracts from blood, brain, kidney, liver, and testis contained an additional metabolite, 3,5-dichloroaniline, which resulted from cleavage of the imide linkage. Cyclopropane-$(COOH)_3$ was presumably present in the tissues and excreta but could not be detected since only the phenyl moiety was labeled.

2. Mutagenicity

Procymidone has been tested in several mutagenicity studies, but all of these have been classified by the Agency as unacceptable. Therefore, mutagenicity testing performed in accordance with the Subdivision F mutagenicity guideline is required to fulfill this data gap.

a) Rec-assay using Bacillus subtilis M45 and H17 strains (MRID #414688-15 and #414688-16): no inhibitory effect on growth in either strain at concentrations of 10 to 10,000 ug/disk. Classification: unacceptable due to improper test design and lack of test results under the activated conditions.

b) Testing with 4 strains of Salmonella typhimurium (TA98, TA100, TA 1535, TA1538) as well as the E. coli WP2 strain (MRID #414688-15 and #414688-16): negative with and without metabolic activation at concentrations of 10 to 10000 ug/plate. Classification: unacceptable because complete procedures were not provided for confirming genotypes of tester strains.

c) Host-mediated assay in mice using G-46 strain of S. typhimurium (MRID #414688-15 and #414688-16): negative with single doses of 1000 and 2000 mg/kg and with double doses of 2 X 200 and 2 X 500 mg/kg. Classification: unacceptable since no preliminary cytotoxicity test results were provided and the high dose was inadequate.

d) Mammalian cells in culture gene mutation assay in V79 cells (MRID #414849-06): negative in the in vitro V-79 cell mutation assay with and without metabolic activation at concentrations of 0.7 to 6 mM. Classification: unacceptable due to an inadequate high dose and the use of improper exposure times.

e) In vivo mammalian cytogenetic assay in mouse bone marrow (MRID #414688-14): not clastogenic in mouse bone marrow assayed 6, 24 and 48 hours after treatment at dose levels of 400 to 1600 mg/kg. Classification: unacceptable due to an inadequate high dose and the use of only one sex.

f) Unscheduled DNA synthesis (UDS) assay in heteroploid epithelial human cells (MRID #414849-06): negative with and without metabolic activation at concentrations of 6 X 10^{-3}M to 6 X 10^{-6}M. Classification: unacceptable due to a lack of cytotoxicity test results and the use of improper exposure times.

g) Sister chromatid exchange (SCE) assay in cultured mouse embryo cells (MRID #414688-17): SCE frequency in the presence and absence of metabolic activation at concentrations of 10^{-4} to 10^{-6}M was not increased. Classification: unacceptable due to the lack of a cell cycle delay study and an inadequate high dose.

3. Acute, Subchronic, and Chronic Toxicity Studies

Procymidone can be classified in Toxicity Category IV for acute oral toxicity and eye and skin irritation, Toxicity Category III for dermal and inhalation toxicity, and the test material was not shown to be a skin sensitizer. None of these studies is required under the conditions of proposed use of Procymidone (import tolerance).

In a subchronic study in dogs (MRID #414849-04), dose levels of 20, 100, and 500 mg/kg/day (capsule) were administered. The NOEL is 100 mg/kg; the LEL is 500 mg/kg, based on increased incidence of emesis (both sexes), diarrhea (females), elevated alkaline phosphatase levels (both sexes), and increased BUN (males).

The administration of Procymidone to rats for 6/9 months (MRID #414849-05) at dose levels of 150, 500, and 1500 ppm in the diet resulted in increased relative liver weight in both sexes at the high-dose level after 6 and 9 months and in females at the mid-dose level after 6 months, increased testes (both absolute and relative) weight after 9 months of dosing at the high-dose, and increased relative brain weight in females after 6 months and in both sexes after 9 months of dosing at the high-dose level. Lower body weights were noted in both sexes at 1500 ppm after 9 months. In the chronic toxicity/carcinogenicity study, a high dose of 2000 ppm was used, which appears appropriate since an MTD may not have been achieved in this subchronic study (strain of rat is different between the two studies). The NOEL is 150 ppm (7.5 mg/kg/day); the LEL is 500 ppm (25 mg/kg/day), based on body- and organ-weight effects.

In a 13-week pilot study (MRID #414849-02) in mice (dose levels of 100, 500, 2500, and 10,000 ppm), a slight depression in body weight was seen in males at the highest dose level. Absolute and relative liver weights were increased in both sexes at the 2500 and 10,000 ppm levels, and absolute and relative kidney weights were depressed in both sexes at the 10,000 ppm level.

Evidence that the liver is the target organ included dose-related increases in liver coagulative necrosis (500, 2500, & 10,000 ppm), coarsely dispersed hepatocyte chromatin (2500 & 10,000 ppm), hepatocyte nuclear enlargement (500, 2500, & 10,000 ppm), cytoplasmic hepatocyte swelling (all dose levels), and multinucleated hepatocytic giant cells (500, 2500, & 10,000 ppm) in either or both sexes. Based on the significant increase in hepatic coagulative necrosis in association with increased liver weights observed at 500 ppm, the selection of 1000 ppm as the high-dose in the mouse carcinogenicity study appears to be justified.

No conclusions can be drawn from a 3-month study in mice fed Procymidone (MRID #414688-12) at dose levels of 50, 150, and 500 ppm, due to the unhealthy state of the test animals. Additionally, in a 6-month study in mice (MRID #414849-03), a dose-related increase in testicular atrophy was observed, but the scientific validity of the study is questioned due to the unhealthy state of the test animals.

In another 6-month feeding study in mice, designed specifically to determine a no-effect levels for testicular atrophy (MRID #414777-02), no evidence of systemic toxicity was observed at dose levels of 10, 30, 100, and 300 ppm. Absolute and relative testes weights were greater in all treatment groups compared with control, but there was no clear evidence from histopathological examination that this was a toxic effect.

A series of studies were conducted on rats and mice designed specifically to examine the possible involvement of gonadotropin disregulation in the production of testicular interstitial cell tumors observed in male rats fed Procymidone. These studies addressed the effects of Procymidone on serum hormone levels, on testicular function, and the affinity of Procymidone to the androgen receptor. The dose levels investigated in these studies include 100, 300, 700, 2000, & 6000 ppm.

The affinity of Procymidone to androgen receptors in the prostate cytosol (MRID #414777-04) was low (0.07% of dihydrotestosterone) in both species. There were inconsistent results in the effects of Procymidone on testes weight, serum testosterone and luteinizing hormone levels among the various studies performed, which do not allow a conclusion as to the "no-hormonal effect" level (300 ppm) determined by the Registrant. Additionally, the Registrant considers the prolonged excessive responsiveness of interstitial cells to stimulation to be the cause

of the induction of interstitial cell tumors in rats, and the transient responsiveness of these cells to stimulation in the mouse to account for the lack of induction of these tumors in the mouse. Although this is plausible, the data submitted do not provide conclusive evidence to support this contention. Of the several parameters measured, only two were significantly different from control at 13 weeks [increased levels of testis testosterone (ng/testis) and pituitary luteinizing hormone (ug/pituitary), and the increases were observed only at 6000 ppm. The highest dietary level in the chronic toxicity/carcinogenicity study in rats was 2000 ppm, and reproductive effects were observed at a dose level of 750 ppm in rats. No data were provided on the responsiveness of interstitial cells to stimulation over a period of time of a long-term study.

4. Developmental and Reproductive Effects

ALpk:APfSD rats were administered Procymidone in the diet at dose levels of 50, 250, & 750 ppm for 12 weeks prior to initial mating, and throughout the production of 2 litters and a second generation, which also produced 2 litters that were maintained for 11 weeks (MRID #414777-08,

Systemic toxicity was observed in adults and pups at 250 and 750 ppm in the form of decreased body-weight gain and food consumption, increased absolute and relative liver weights in the males, increased testes weights and combined and adjusted testes volume, and decreased pup prostate and epididymal absolute and relative weights. This toxicity was further corroborated by evidence of macroscopic and microscopic changes in the liver and male external genitalia. Reproduction/developmental toxicity was evident at the high dose, which caused abnormalities of external genitalia (reduced ano-genital distance and hypospadias) in F_1 and F_2 males and infertility in F_1 males, presumed to be a consequence of malformation induced by in utero exposure during late gestation. Minor histological changes in pituitary and a reduction in size and weight of the accessory sex organs were also observed in these animals. There were no similar effects at any other dose level or on F_0 males. A tentative systemic NOEL is set at 50 ppm; the tentative LEL at 250 ppm, based on body weight and organ weight effects. A tentative reproductive NOEL is set at 250 ppm; a tentative LEL at 750 ppm, based on abnormalities of external genitalia in F_1 and F_2 males and infertility in F_1 males.

NOTE: A reduction in ano-genital length was also observed following exposure to vinclozolin, a structurally-related compound.

There are two developmental toxicity studies. In the rat study (MRID #414777-06), Procymidone was administered orally in corn oil at dose levels of 30, 100, and 300 mg/kg on gestation days 6 through 15. There was no evidence of maternal or developmental toxicity. The dose levels used were not high enough to adequately

assess the potential of developmental and/or maternal toxicity.

In the rabbit study (MRID #414777-07), dose levels of 30, 150, 750, and 1000 mg/kg did not induce maternal toxicity nor was developmental toxicity evident from the investigation of deaths/ resorptions, altered growth, developmental anomalies and malformations. The maternal NOEL and LEL are greater than 1000 mg/kg (limit dose). The developmental NOEL and LEL are greater than 1000 mg/kg also.

5. Structure-Activity Correlations

Procymidone is structurally similar to Iprodione and Vinclozolin (see structures following). Although Procymidone appears to be structurally related to Iprodione and Vinclozolin, their chemical properties may differ. Procymidone, being a cyclic imide, may have some acylating activity and would be expected to be less stable than Iprodione and Vinclozolin (the additional heteroatom should make the ring more stable than Procymidone). Also, the vinyl group in Vinclozolin is a potential pro-electrophile.

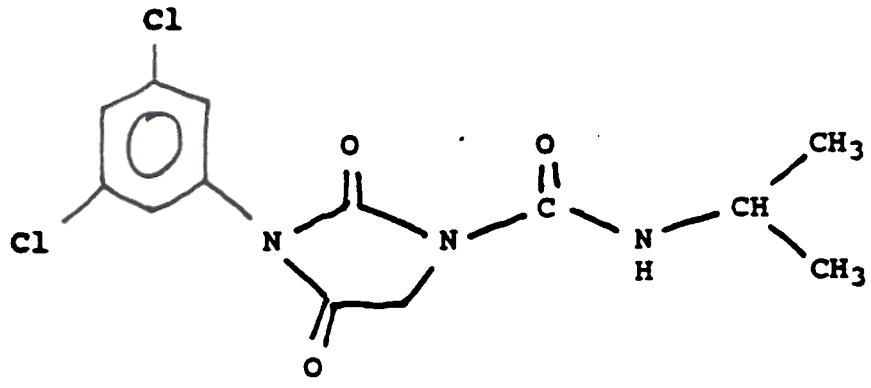
a) Iprodione: 3-(3,5-dichlorophenyl)-N-(1-methylethyl)-2,4-dioxo-1-imidazoline carboxamide (a fungicide). Iprodione has been tested in both the rat chronic feeding/carcinogenicity and mouse carcinogenicity studies. In the rat study, there was no carcinogenic response; the NOEL for systemic effects was set at >1000 ppm (HDT; 50 mg/kg). The other doses were 125 and 250 ppm. The mouse study was also negative for carcinogenicity, with the systemic NOEL set at 1250 ppm (HDT; 62.5 mg/kg); other doses were 200 and 500 ppm. It is to be noted that both the mouse and rat studies were found inadequate during the FIFRA Phase 2 review. There are several acceptable mutagenic studies on Iprodione. Negative results were obtained in a mouse dominant lethal test, a forward mutation (CHO/HGPRT) assay with and without activation, and in the sister chromatid exchange assay with CHO cells with and without activation. Iprodione was positive in the DNA damage/repair assay with B. subtilis (rec assay) at the highest and lowest concentrations used (1670 and 20.6 ug/disc) without metabolic activation.

b) Vinclozolin or Ronilan: 3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidin-2,4-dion is a fungicide, bactericide, and wood preservative. It has been tested in both the rat chronic toxicity feeding/carcinogenicity and mouse carcinogenicity studies. In the rat (26 months) study, there was no evidence of a carcinogenic effect. The systemic NOEL was set at 486 ppm (24.3 mg/kg), the LEL at 1458 ppm (72.9 mg/kg), based on body weight reduction and reduced serum bilirubin. Doses tested were 162, 486, & 1458 ppm. In the mouse (26 months) study, the systemic NOEL was set at 486 ppm (72.7 mg/kg), the LEL at 1458 ppm (218.7 mg/kg), based on decreased body weight in the males. There was an increase in

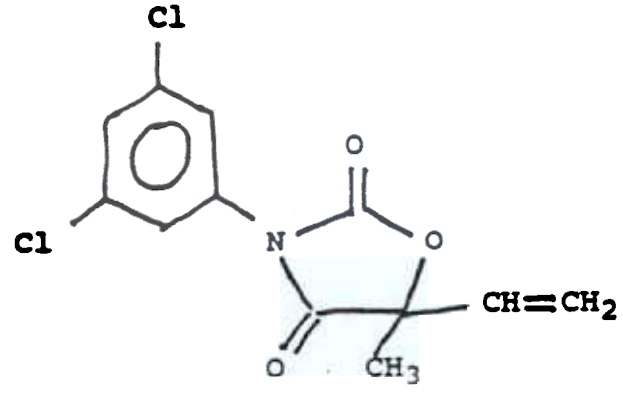
leukemia/lymphoma in males, an apparent dose-related increase in lung adenoma in females, and a low but increased incidence of liver adenomas at the HDT in males. The Peer Review Committee concluded that the mouse carcinogenicity, the rat chronic toxicity/ carcinogenicity, and mutagenicity studies did not support the finding that the test material was carcinogenic. It is to be noted that both studies are being repeated.

Vinclozolin has been tested in several mutagenicity studies. Positive and negative results have been obtained in several Salmonella assays. Negative results have been obtained in a host-mediated assay, a CHO/HGPRT assay, a mouse lymphoma assay, an UDS/primary rat hepatocytes assay and a SCE assay in hamsters. There is a data gap for testing in the structural chromosomal aberrations category.

Structures of Analogues:



Iprodione



Vinclozolin

c) 3,5-Dichloroaniline is a metabolite of Procymidone. Very little data were available on this compound. It does not induce UDS in primary rat hepatocytes as reported in the literature. It may have a carcinogenicity concern based on analogy to p-chloroaniline which has equivocal evidence for carcinogenicity (NTP Technical Report No. 189) for the circulatory system in male and female mice and for the spleen in male rats; however, these are different targets than those seen with Procymidone. p-Chloroaniline is positive in the Salmonella assay (Procymidone appears negative in this assay - see above).

F. Weight of Evidence Considerations:

The Committee considered the following facts regarding the toxicology data on Procymidone to be of importance in a weight-of-the-evidence determination of carcinogenic potential.

1. The administration of Procymidone was associated with an increased incidence of testicular interstitial cell tumors in male Osborne Mendel rats. At dietary levels of 1000 and 2000 ppm Procymidone, the incidence of testicular tumors was significantly increased (dose-related) above control incidence, and there was a significant trend. Additionally, the incidence of testicular interstitial cell hyperplasia was significantly increased at these two dose levels.

2. Pituitary adenomas were significantly increased at the 1000 and 2000 ppm dose levels in female rats, and there was a significant positive trend.

3. Male mice had a significant positive trend in hepatoblastomas (a rare variant of hepatocellular carcinoma) with increasing dose levels of Procymidone. While pair-wise statistical significance was not obtained, it was noted that this tumor type did not show up in the historical database (outside of the concurrent control). Also, it was considered that the top dosing in this study could have been significantly higher, thus possibly increasing the induced incidence.

4. At the high dose (1000 ppm), female mice demonstrated a significant increase in both hepatocellular adenomas and in combined adenoma and/or carcinoma. Additionally, there was a significant positive trend in hepatocellular adenomas and in combined adenoma and/or carcinoma. Also, it was considered that the top dosing in this study could have been significantly higher, thus possibly increasing the induced incidence.

5. Although none of the mutagenicity studies on Procymidone indicate a genotoxic effect, all are classified as unacceptable for various deficiencies (e.g. study performance, inadequate dosing). It is suggested that these studies do not indicate a mutagenicity concern; however, full confidence cannot be placed in the apparent negative findings since these studies have deficiencies based on current standards.

6. The two major analogues, Iprodione and Vinclozolin, provide little SAR support for the carcinogenicity of Procymidone. The Iprodione studies were inadequate (although reported negative) and the Peer Review Committee concluded that the evidence for Vinclozolin does not support a carcinogenicity concern. Also, it was pointed out that the chemical properties of these three compounds may differ. The available evidence for the metabolite 3,5-dichloroaniline also provides little support for a

carcinogenicity concern (e.g. its analogue p-chloroaniline has a different target).

7. Procymidone was considered a reproductive/developmental toxicant based on the rat multigeneration study where abnormalities of external genitalia and infertility of males were observed. It was noted that the non-cancer health effects, including adverse reproductive effects, occur much earlier than the reported carcinogenic effects and at quite low doses. It is recommended that the evidence for reproduction and developmental effects be reviewed by the Health Effects Division's Reproduction and Developmental Peer Review Committee.

8. The Registrant performed special studies in rats and mice to support their contention that the noted testicular tumors were the result of prolonged hormonal stimulation of the interstitial cells. These studies suggested that Procymidone increased serum levels of luteinizing hormone and testosterone and that Procymidone has a low affinity for androgen receptors. They did not demonstrate responsiveness of interstitial cells to stimulation in a long-term study.

The Peer Review Committee noted that the targets associated with Procymidone exposure (pituitary, ovaries, testicular tissues) as well as the possible effect on LH and testosterone levels may be a reflection of some underlying mechanism (e.g. possible steroidal effects on testes). However, the Committee agreed that the data presented were not conclusive to establish hormonal effects as the basis for tumor induction.

9. The evidence establishes the liver as a major site of target-organ toxicity in both rodent species. This is based on liver tumor incidences in mice as well as non-neoplastic liver lesions in both sexes of rats and mice. Any differences in the degree of toxicity between the species may be due to differences in metabolism rates.

10. Possible interaction or synergism between Procymidone and alcohol in wine products containing Procymidone was mentioned and may need to be investigated. This was considered due to observed liver toxicity and reproductive effects associated with both compounds.

G. Classification of Carcinogenic Potential:

Criteria contained in the EPA Guidelines [FR51: 33992-34003, 1986] for classifying a carcinogen were considered.

The Peer Review Committee voted nine votes to classify Procymidone as a Group B_2 - Probable Human Carcinogen and eight votes to classify Procymidone as a Group C - Possible Human Carcinogen.

The B_2 classification was based on the statistically significant increasing trend and pair-wise increase in interstitial cell adenomas in male rats, pituitary adenomas in females rats, and liver adenomas and combined adenomas/carcinomas in female mice. Additionally, a rare variant of hepatocellular carcinoma, hepatoblastoma, had a significantly increasing trend in male mice. The hepatoblastoma rate at the top dose was well outside the historical control range. While the tumor incidences in mice were not greatly elevated, the effects were seen at doses well below an adequate top dose. The available evidence for a mechanism involving altered hormonal influences was not conclusive. Also, potential acylating activity as a cyclic imide was considered.

The C classification was supported by the same evidence as above, but with a different consideration. Although there were significant increases in tumors in two species (both sexes of rat and in the female mouse), these were primarily benign. The one malignant tumor, hepatoblastoma in male mice, occurred with a significant positive trend, but was not significantly increased in a pair-wise comparison with controls. Also, the hepatoblastomas were late occurring, first appearing at week 105 at the highest dose. The female mouse liver tumors were found only at the highest dose with a pair-wise comparison to controls 0.03 to 0.04. The combined female mouse tumor incidence, consisting mainly of benign tumors, was just outside historical controls. The female rat pituitary tumors were benign and considered common in aging rats. It was found difficult to ascribe biological significance to this increase in pituitary tumors. There was a lack of support from genotoxicity evidence and SAR considerations. Finally, while the available evidence for a mechanism involving altered hormonal influences was not conclusive, based on the effects seen in several endocrine targets, this mechanism was considered possible and additional studies were encouraged.

For the purpose of risk characterization, a low dose extrapolation model applied to the experimental animal tumor data was overwhelmingly recommended for quantification of human risk (Q_1^*) regardless of the classification. A quantification of [redacted] potential is recommended for the testicular tumors in male rats and for the liver tumors in female mice. These would be performed to compare the risk estimates as well as to examine the possibility of combining the risk estimates into one risk estimate.

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Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, *p,p'*-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat †

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Antiandrogenic chemicals alter sexual differentiation by a variety of mechanisms, and as a consequence, they induce different profiles of effects. For example, *in utero* treatment with the androgen receptor (AR) antagonist, flutamide, produces ventral prostate agenesis and testicular nondescent, while in contrast, finasteride, an inhibitor of 5α-dihydrotestosterone (DHT) synthesis, rarely, if ever, induces such malformations. In this regard, it was recently proposed that dibutyl phthalate (DBP) alters reproductive development by a different mechanism of action than flutamide or vinclozolin (V), which are AR antagonists, because the male offsprings display an unusually high incidence of testicular and epididymal alterations—effects rarely seen after *in utero* flutamide or V treatment. In this study, we present original data describing the reproductive effects of 10 known or suspected anti-androgens, including a Leydig cell toxicant ethane dimethane sulphonate (EDS, 50 mg kg^{-1} day^{-1}), linuron (L, 100 mg kg^{-1} day^{-1}), *p,p'*-DDE (100 mg kg^{-1} day^{-1}), ketoconazole (12–50 mg kg^{-1} day^{-1}), procymidone (P, 100 mg kg^{-1} day^{-1}), chlozolinate (100 mg kg^{-1} day^{-1}), iprodione (100 mg kg^{-1} day^{-1}), DBP (500 mg kg^{-1} day^{-1}), diethylhexyl phthalate (DEHP, 750 mg kg^{-1} day^{-1}), and polychlorinated biphenyl (PCB) congener no. 169 (single dose of 1.8 mg kg^{-1}). Our analysis indicates that the chemicals discussed here can be clustered into three or four separate groups, based on the resulting profiles of reproductive effects. Vinclozolin, P, and DDE, known AR ligands, produce similar profiles of toxicity. However, *p,p'*-DDE is less potent in this regard. DBP and DEHP produce a profile distinct from the above AR ligands. Male offsprings display a higher incidence of epididymal and testicular lesions than generally seen with flutamide, P, or V even at high dosage levels. Linuron treatment induced a level of external effects consistent with its low affinity for AR [reduced anogenital distance (AGD), retained nipples, and a low incidence of hypospadias]. However, L treatment also induced an unanticipated degree of malformed epididymides and testis atrophy. In fact, the profile of effects induced by L was similar to that seen with DBP. These results suggest that L may display several mechanisms of endocrine toxicity, one of which involves AR binding. Chlozolinate and iprodione did not produce any signs of maternal or fetal endocrine toxicity at 100 mg kg^{-1} day^{-1}. EDS produced severe maternal toxicity and a 45% reduction in size at birth, which resulted in the death of all neonates by 5 days of age. However, EDS only reduced AGD in male pups by 15%. Ketoconazole did not demasculinize or feminize males but rather displayed anti-hormonal activities, apparently by inhibiting ovarian hormone synthesis, which resulted in delayed delivery and whole litter loss. In summary, the above *in vivo* data suggest that the chemicals we studied alter male sexual differentiation via different mechanisms. The anti-androgens V, P, and *p,p'*-DDE produce flutamide-like profiles that are distinct from those seen with DBP, DEHP, and L. The effects of PCB 169 bear little resemblance to those of any known anti-androgen. Only in depth *in vitro* studies will reveal the degree to which one can rely upon *in vivo* studies, like those presented here, to predict the cellular and molecular mechanisms of developmental toxicity.

Keywords: *anti-androgen, developmental reproductive toxicology, endocrine disruptor, inhibition of steroid synthesis, pesticides, phthalate, sex reversal, steroid receptors.*

1. Abbreviations: AGD, anogenital distance; Ah, aryl hydrocarbon; ANOVA, analysis of variance; AR, androgen receptor; DBP, dibutyl phthalate; DEHP, diethylhexyl phthalate; DHT, 5α-dihydrotestosterone; EDS, ethane dimethane sulphonate; EDSTAC, Endocrine Disruptor Screening and Testing Advisory Committee; EPA, Environmental Protection Agency; GD, gestational day; L, linuron; LE, Long–Evans; LH, luteinizing hormone; LQ, lordosis quotient; NOAEL, no-observed-adverse-effect level; P, procymidone; PCB, polychlorinated biphenyl; PCDF, polychlorinated dibenzofuran; PHI, pseudohermaphroditism index; PND, postnatal day; s.c., subcutaneous; SD, Sprague–Dawley; T, testosterone; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; V, vinclozolin

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Introduction

Exposure to 'endocrine-disrupting' pesticides, toxic substances, and drugs during critical stages of life can produce permanent alterations of vertebrate reproductive function. Several cases of clear-cut, cause-and-effect relationships exist between field exposures and adverse reproductive outcomes in fish, birds, and mammals (Colborn and Clement, 1992; Ankley and Giesy, 1998). In addition, there are numerous instances where a significant correlation exists between toxicant exposure in the field and impaired reproduction. Human reproduction has been affected by chemicals of this nature as well [drugs and polychlorinated biphenyls (PCBs)], with reported effects ranging from declining sperm counts (Carlsen et al., 1992) to an increased female/male sex ratio (Davis et al., 1998).

Recently, interest in testing chemicals for endocrine-disrupting activity has intensified. New laws require the US Environmental Protection Agency (EPA) to test pesticides for estrogen-like effects or other endocrine effects, as designated by the EPA Administrator. It is critical to recognize that pesticides alter reproductive function via several mechanisms of toxicity besides estrogenicity and to identify methods useful for testing for these activities.

Administration of androgenic substances profoundly alters rodent sexual differentiation. Females resemble males by having an increased anogenital distance (AGD) and enlarged phallus, and they have abnormalities of the uterus, vagina, and mammary glands. Paradoxically, Wilson and Wilson (1943) demonstrated that male rats treated neonatally with high doses of testosterone (T) had small hypospermatogenic testes and reduced accessory sex gland and epididymal weights. Progestins, which display weak affinity for the androgen receptor (AR), alter male reproductive differentiation in an antiandrogenic manner, causing hypospadias, ambiguous genitalia, and occasional testicular atrophy (Schardein, 1993). Some of these compounds, including dimethisterone, hydroxyprogesterone, medroxyprogesterone, norethindrone, and progesterone produce a low incidence of malformations in humans. Similar effects have been obtained with progestins in developing male rodents and monkeys (Prahalada et al., 1985). The pharmaceuticals, flutamide, which inhibits T and 5α-dihydrotestosterone (DHT) binding to the intracellular AR, and finasteride, which inhibits DHT synthesis by acting as a 5α-reductase inhibitor (Imperato-McGinley et al., 1992), also demasculinize the external genitalia of male rodent offspring.

We found that several pesticides act as 'environmental anti-androgens' with sufficient potency to induce hypospadias and ambiguous genitalia. The fungicide, vinclozolin (V), inhibits sexual differentiation as a consequence of antiandrogenic action (Gray et al., 1994). When rats are exposed to V (100 and 200 mg kg^{-1} day^{-1}) during late gestation and early lactation, AGD in male offspring is reduced to a female-like size, and the male progeny displays retained nipples, hypospadias, ectopic undescended testes, and agenesis of the prostate. Dosage levels as low as 3 mg kg^{-1} day^{-1} reduce AGD (Gray et al., this issue). *In vitro* studies indicate that V metabolites, M1 and M2, but not V itself, block sexual differentiation by acting as competitive AR antagonists (Kelce et al., 1994). Some other pesticides that display antiandrogenic activity are the persistent DDT metabolite *p,p'*-DDE (Kelce et al., 1994) and procymidone (P) (Gray and Kelce, 1996; Ostby et al., this issue).

The profile produced by fetal exposure to AR antagonists like flutamide and P differs considerably from that produced by inhibition of DHT synthesis with 5α-reductase inhibitors like finasteride. Of these two drugs, only flutamide treatment induces high incidences of ventral prostate agenesis and testicular nondescent. In this regard, it has been proposed that a careful examination of the profile of *in vivo* effects of a chemical that demasculinizes and feminizes the fetal male can enable one to formulate a hypothesis about the cellular and molecular mechanism of antiandrogenic action. For example, dibutyl phthalate (DBP) produces a profile that is distinct from that produced by chemicals that act solely as AR antagonists (Mylchreest et al., 1998). DBP induces high incidences of epididymal agenesis and testicular abnormalities, while treatment with flutamide or V at dosage levels that induce hypospadias in 100% of the males has much less of an effect on the epididymides and testes. Hence, it is unlikely that DBP acts solely as an AR antagonist, if it binds AR at all.

With this in mind, it was our objective to describe the profile of effects of several potentially antiandrogenic chemicals. In the current study, we present original data describing the reproductive effects of 10 known or suspected anti-androgens, including linuron (L, 100 mg kg^{-1} day^{-1}), *p,p'*-DDE (100 mg kg^{-1} day^{-1}), P (100 mg kg^{-1} day^{-1}), iprodione (100 mg kg^{-1} day^{-1}), chlozolinate (100 mg kg^{-1} day^{-1}), ethane dimethane sulphonate (EDS, 50 mg kg^{-1} day^{-1}), ketoconazole (12–50 mg kg^{-1} day^{-1}), DBP (500 mg kg^{-1} day^{-1}), diethylhexyl phthalate (DEHP, 750 mg kg^{-1} day^{-1}), and PCB congener no. 169 (single dose of 1.8 mg kg^{-1}). We expected *p,p'*-DDE and L to produce profiles that resembled flutamide, V, and P because they are all known AR ligands. We wanted to determine if chlozolinate and iprodione, two other dicar-

boximide fungicides like V and P, acted as anti-androgens *in vivo*. We included ketoconazole and EDS in this study because they inhibit the synthesis of T (ketoconazole is a cytochrome P-450 enzyme inhibitor and EDS kills Leydig cells). We suspected that ketoconazole and EDS might alter differentiation of T-dependent tissues to a greater degree than do the AR ligands. In the current investigation, we also wanted to confirm the reported effects of DBP on the male rat and extend these observations to another phthalate, DEHP. The aryl hydrocarbon (Ah) receptor agonist, PCB 169, is included here to determine if it alters reproductive development in the rat in a manner that resembles 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Gray and Ostby, 1995; Gray et al., 1995a, 1997a,b) and to determine to what degree these effects resemble the action of known antiandrogenic substances.

Background information on the specific chemicals investigated in the study

Linuron

Linuron is a urea-based herbicide with an acute oral LD_{50} for rats of 4000 mg kg^{-1}. In a 2-year feeding trial, L increased the incidence of testicular tumors in rats at 125 and 625 mg kg^{-1} day^{-1} in the diet (Cook et al., 1993). It was proposed that L might produce this effect via an endocrine alteration (Rehnberg et al., 1988; Cook et al., 1993), because prolonged luteinizing hormone (LH) hyperstimulation of rat Leydig cells results in hyperplasia and adenoma formation. Consistent with this proposed mechanism of action, Cook et al. (1993) and Waller et al. (1996) demonstrated that L displayed weak affinity for the AR. However, *in vivo* short-term dosing with L produced minimal effects on pituitary and testicular serum hormone levels, and it failed to reduce androgen-dependent sex accessory gland size in immature and adult male rats at dosage levels that were not overtly toxic (Rehnberg et al., 1988; Cook et al., 1993).

Phthalates, DBP and DEHP

In contrast to V, P, and *p,p'*-DDE, the phthalates represent a class of toxicants that alter reproductive development via unknown mechanisms. Although there are numerous publications on the effects of phthalates on testicular function in young adult male rats (Foster et al., 1980), little attention has been paid to the effects of this class of chemicals on sexual differentiation. One recent publication indicated that perinatal DBP altered development of the male reproductive tract (Wine et al., 1997), causing underdevelopment and agenesis of the epididymis and suggested that such effects may have resulted from the estrogenic action of DBP. While DBP is considered by many to be an 'environmental estrogen' because it is estrogenic *in vitro* (Jobling et al., 1995), Mylchreest et al. (1998) hypothesized that DBP was antiandrogenic because it induced reproductive tract malformations in several androgen-dependent tissues in male progeny after prenatal DBP treatment, including reduced AGD, hypospadias, undescended testes, and cleft phallus. However, based on the observation of a high incidence of epididymal and testicular lesions in DBP-treated male offspring, they proposed that DBP did not act via the same mechanism as V, P, or *p,p'*-DDE (Mylchreest et al., 1998).

Chlozolinate and Iprodione

Chlozolinate and iprodione are dicarboximide fungicides, similar in structure to the anti-androgens V and P. However, there are no published studies reporting whether or not chlozolinate and iprodione display antiandrogenic activity *in vivo*. Hence, these two pesticides were included in the current investigation to determine if other members of this class of fungicides produced endocrine alterations that resembled the effects of V.

Procymidone

Procymidone is a dicarboximide fungicide structurally related to the well-characterized antiandrogenic fungicide, V. Vinclozolin metabolites, M1 and M2, bind both rat and human androgen receptors and act as AR antagonists. M1 and M2 block androgen-dependent gene expression *in vivo* and *in vitro* by inhibiting androgen-induced AR–DNA binding (Kelce et al., 1994; Wong et al., 1995). The antiandrogenic activity of P has been much less thoroughly characterized *in vivo* and *in vitro* than V. Competitive binding studies show that P displays weak affinity for AR (Hosokawa et al., 1993a; Waller et al., 1996). Ostby et al. (this issue) determined that P altered sexual differentiation at dosage levels as low as 25 mg kg^{-1} day^{-1}, but failed to determine a NOAEL (no-observed-adverse-effect level). Perinatal administration of P [25–200 mg kg^{-1} day^{-1} on gestational day (GD) 14 to day 3 after birth] reduced AGD, induced retained nipples, hypospadias, cleft phallus, and a vaginal pouch, and reduced sex accessory gland size in male Long–Evans (LE) hooded rat offspring. In addition, treated males displayed increased incidences of inflammation of the prostate and seminal vesicles at 50 mg kg^{-1} day^{-1} and above when necropsied at 16 months of age. Such data are likely to be critical in the risk assessment of P, because P produces reproductive malformations in the fetal male at dosage levels (50 mg kg^{-1} day^{-1} and above) that have little effect on the reproductive tract of the adult male rat (Hosokawa et al., 1993b).

p,p'-DDE

We have shown that *p,p'*-DDE displays antiandrogenic activity both *in vivo* and *in vitro* (Kelce et al., 1995). *In vivo*, *p,p'*-DDE reduces AGD and induces retained nip-

ples, and treated males have smaller ventral prostates (Kelce et al., 1995). However, hypospadias was not observed in *p,p'*-DDE-treated males. *p,p'*-DDE also displays antiandrogenic activity when administered during puberty (Kelce et al., 1995), producing a delay in androgen-dependent preputial separation. *In vitro*, *p,p'*-DDE binds to the AR and prevents DHT-induced transcriptional activation in cells transfected with the human AR. When *p,p'*-DDT, which is also antiandrogenic (Kelce et al., 1995), was administered to Dutch Belted rabbits during gestation (does-treated) and lactation (pups treated) at dosage levels well below those used here (25 mg kg^{-1}: does; 10 mg kg^{-1}: pups; 1 time/$week^{-1}$), reproductive abnormalities were displayed by male offsprings (Veeramachaneni et al., 1995). Infantile exposure alone resulted in delays of up to 6 weeks in testicular descent. When combined with gestational exposures, uni/bilateral cryptorchidism also was observed. Serum levels in offspring were 208 ppb *p,p'*-DDT and 38 ppb *p,p'*-DDE. The identification of *p,p'*-DDE and *p,p'*-DDT as anti-androgens may explain some of the effects of this pesticide on wildlife. The high incidence of undescended testes in the Florida panther could be the result of the high levels of *p,p'*-DDE in the food chain (Facemire et al., 1995, 1997).

Our earlier study with *p,p'*-DDE emphasized the neonatal alterations seen in the LE hooded rat after *in utero* exposure to 100 mg kg^{-1} day^{-1} on days 14–18 of pregnancy. The current experiment with *p,p'*-DDE was designed to examine the intrauterine effects of this compound in both Sprague–Dawley (SD) and LE hooded rats, with greater emphasis on identification of permanent alterations than in our earlier study.

Putative Androgenic Effects of PCB 169, an Ah Receptor Agonist

Exposure to Ah receptor agonists such as TCDD, PCBs, and polychlorinated dibenzofurans (PCDFs) is causally linked to developmental/reproductive toxicity in humans, primates, rodents, mink, fish, and other wildlife species (Golub et al., 1991; Bjerke and Peterson, 1994; Bjerke et al., 1994a,b; Gray and Ostby, 1995; Gray et al., 1995a, 1997a,b; Ankley and Giesy, 1998; Giesy and Snyder, 1998). *In utero* exposure to a mixture of PCB/PCDFs in 1978–1979 in Taiwan resulted in 'Yu Cheng' illness in children characterized by hyperpigmentation, nail deformities, and poorer cognitive and behavioral development (Lai et al., 1994), and males had a shorter penis at 11–14 years of age (Guo et al., 1993). TCDD, and other toxicants with similar structure, bind with high affinity to an intracellular steroid hormone-like receptor, termed the Ah receptor, forming a complex that acts as a transcriptional factor by binding to specific dioxin response elements on specific genes. Some, if not all, of the toxicity of TCDD appear to result from activation of the Ah receptor. TCDD is an 'endocrine disruptor' that acts on multiple components of the endocrine axis (Birnbaum, 1994). TCDD exposure alters the levels of many hormones, growth factors, and their receptors as well as hormone synthesis. For these reasons, it is arguable whether TCDD should be classified as an anti-androgen. Initially, it was reported that fetal and neonatal T levels were suppressed by TCDD treatment and that AGD was shortened in male neonates. However, replication of these effects remains elusive and the classification of these compounds as 'anti-androgens' is controversial. Although the precise endocrine mechanism of toxicity has not been resolved for TCDD, it is clear that exposure to a single low dose of TCDD ranging from 50 ng to 2 μg kg^{-1} during sexual differentiation of the rat or Syrian hamster results in a number of unusual reproductive alterations in males and females (Gray et al., 1997a,b). Since the early 1970s, it has been known that exposure to very low doses of TCDD produces infertility in rodent progeny (Khera and Ruddick, 1973; Murray et al., 1979; Mably et al., 1992a,b,c; Bjerke and Peterson, 1994; Bjerke et al., 1994a,b; Gray and Ostby, 1995; Gray et al., 1995a). In TCDD-exposed male offspring, puberty is delayed and ejaculated and epididymal sperm numbers are reduced, with less effect on testicular sperm production. Since T levels are not reduced following perinatal TCDD exposure, the alterations in these tissues are not likely to have resulted from an alteration of the androgenic hormonal status of the male offspring.

In contrast to TCDD, known antiandrogenic chemicals do not cause malformations in the female offspring. In female rat offspring, 0.2 to 1 μg TCDD kg^{-1} on GD 15 induces clefting of the phallus with a mild degree of hypospadias and a permanent 'thread' of tissue across the opening of the vagina (Gray and Ostby, 1995; Gray et al., 1997b). The lower dosage levels of TCDD result in fetal concentrations of about 10 ppt TCDD (Hurst et al., 1998). Female progeny, treated earlier in pregnancy with 1 μg TCDD kg^{-1}, displayed reduced fecundity, a high incidence of constant estrous, and cystic endometrial hyperplasia at middle-age. One question that needs to be addressed is whether TCDD is a 'transplacental' carcinogen. To date, we have observed two rare carcinomas in aging female rats exposed to TCDD *in utero*, with a single case each of a rare uterine and ovarian carcinoma. Female hamsters, treated on day 11 of gestation with 2 μg TCDD kg^{-1}, also display clitoric clefting and reduced fertility, but they do not display the vaginal 'thread.'

The PCB congener 169 is an Ah receptor agonist with a toxic equivalency factor of about 0.001. Administration of this congener on GD 1 at 1.8 mg kg^{-1} reduces fertility in the offspring (Smits-van Prooije et al., 1993). However, a

detailed examination of the reproductive system was not included in the original study. In this regard, the present experiment was designed to determine if PCB 169 treatment during pregnancy alters reproductive development of LE hooded male and female rats and, if so, whether the profile of reproductive effects is similar to that produced by TCDD and whether some of the androgen sensitive tissues (AGD, nipples, areolas) are altered.

SIS (Steroidogenic-Inhibiting Substances): Ketoconazole

Interaction with the AR is only one of many mechanisms of antiandrogenic action. Chemicals also can disrupt the hormonal regulation of reproduction by inhibiting the synthesis of the steroid hormones. Goldman et al. (1976) produced male pseudohermaphroditism in rats with inhibitors of steroid 17α-hydroxylase and C17–20 lyase. In this regard, several classes of fungicides disrupt fungal growth and mammalian steroidogenesis by blocking enzymes in the sterol/steroidogenic pathway. In fact, several of these 'fungicides' are of sufficient potency that they are used to control endocrinopathies in humans. For these chemicals, the profiles of effects vary greatly, depending on which enzyme in the pathway is most affected. The fungicide, fenarimol, has a pronounced effect on estradiol synthesis, resulting in a loss of male mating behavior and delays in parturition (Gray and Ostby, 1998).

Ketoconazole is a well-studied drug that blocks fungal growth and T synthesis in humans and rodents by inhibiting the activity of cytochrome P-450 enzymes. Schurmeyer and Nieschlag (1984) demonstrated that ketoconazole and other imidazole fungicides inhibited T production in males, while Pepper et al. (1990) reported that ketoconazole was useful in the treatment of ovarian hyperandrogenism in women.

Ethane Dimethane Sulphonate (EDS)

Ethane-1,2-dimethane sulfonate is an alkylating sulfonic acid ester that selectively kills adult and fetal populations of Leydig cells in the rat testis (Bartlett et al., 1986; Kerr et al., 1987; Morris et al., 1988a; Zaidi et al., 1988). The temporal endocrine and histological changes, ranging from a few hours after injection to 70 days after EDS treatment at 75 to 100 mg kg^{-1}, have been thoroughly described for the adult male rat (Edwards et al., 1970; Bartlett et al., 1986; Morris et al., 1986; Drummond et al., 1988; Morris et al., 1988b; Gray et al., 1995b). In the rat, serum T levels begin to fall by 6 to 24 h after dosing, and by 9 to 10 days, T is undetectable. At this latter time, Leydig cells are absent from the testis of the adult rat, T levels are undetectable in the serum, interstitial fluid and seminiferous tubular fluid of the testis are reduced, and, consequently, spermatogenesis and fertility are reduced. EDS also is effective when administered orally by gavage or in drinking water at relatively low dosage levels (Jackson, 1973). The present study was designed to evaluate the effect of EDS on fetal and neonatal rat sexual differentiation. We hypothesized that if EDS was cytotoxic to fetal Leydig cells, then the male offspring would display reduced AGD and other antiandrogenic effects.

General methods

Animals

Pregnant LE hooded and SD rats were purchased from Charles River Breeding Laboratory, Raleigh NC, on day 2 of gestation and were housed individually in clear plastic cages (20 cm × 2 cm × 547 cm) with heat- treated (to eliminate resins that induce liver enzymes) laboratory-grade pine shavings (Northeastern Products, Warrensburg, NY) as bedding. Animals were maintained on a special diet during gestation and lactation (Purina Chow 5008), after which the pups were switched to Purina Rat Chow (5001) and tap water *ad libitum*. They were kept in a room with a 14:10 hour photoperiod (L/D, lights off at 1100 EST), a temperature of 20–24°C, and a relative humidity of 40–50%. Maternal/pup viability and growth were monitored throughout the experiments.

Multigenerational Studies

The multigenerational studies presented here on L and DBP were conducted using a modified Alternative Reproductive Test protocol developed in our laboratory (Gray et al., 1988, 1989, 1990; Zenick et al., 1994) using LE Hooded rats. In this protocol, the P0 generation is exposed by daily gavage from weaning, through puberty, young adulthood, mating, and lactation. F1 pups are evaluated through mating, but they are exposed only via the dam during gestation and lactation. In these studies, 21-day old pups (the P0) are assigned to treatments in a manner that provides them with equal means and variances in body weight. Treatment groups typically consist of 16–24 animals (8–12 of each sex). Treatments are initiated at weaning (day 21) by gavage (5 μl of corn oil [g body weight]$^{-1}$) on a mg kg^{-1} day^{-1} basis. As the animals grow, the volume administered is reduced to 2.5 μl (g body weight)$^{-1}$. This dosing regimen includes twice weekly dose adjustments based on weight changes throughout the study. In P0 females, dosing is continued through postpartum day 20, while males are dosed daily from weaning until necropsy. In the L multigenerational study, both male and female rats from the P0 generation were treated with 0 ($n = 12$/sex), 10, 20, or 40 mg L kg^{-1} day^{-1} ($n = 8$ sex^{-1} in each L group) and were mated with similarly treated animals to generate the F1. In DBP studies, both male and female rats were dosed with DBP (10–12 sex^{-1} $group^{-1}$). In the first DBP study, dosage levels of 0, 250,

500, and 1000 (males only at 1000) mg kg^{-1} day^{-1} were used, while other DBP studies dosed with 0 and 500 mg kg^{-1} day^{-1} or 0 and 1000 mg kg^{-1} day^{-1}. When the P0 DBP animals were mated to produce the F1, treated animals were paired with untreated controls. These dosage levels were selected because our previous pilot study found that treatment with 1000 mg kg^{-1} day^{-1} results in total infertility in both male and female rats in the P0 generation.

These multigenerational studies include determination of the age of puberty in P0 male and female rats, estrous cyclicity, and fertility, and the reproductive system is evaluated at necropsy (for detailed methods, see Gray et al., 1988, 1989, 1990; Zenick et al., 1994). When the P0 males are necropsied, serum is collected for hormonal analyses, organ weights are determined (body, testes, liver, kidney, adrenal, seminal vesicle plus coagulating glands with fluids, epididymis, and pituitary), and testicular histology, cauda epididymal sperm, and testicular sonication-resistant spermatid head numbers are examined. When the F1 rat pups reach 25 days of age, the P0 dams are euthanized using CO_2 and necropsied for determination of body, liver, kidney, adrenal, pituitary, and ovary weights and the number of implantation scars.

Litter sizes and weights of the F1 are taken throughout the study. After puberty, F1 male and female rats (exposed only indirectly via the dam during gestation and lactation) are randomly selected from the highest dose with sufficient numbers of surviving pups for fertility assessment under continuous mating conditions ($n = 16$ group^{-1} sex^{-1}). F2 pups are counted and removed at birth. All surplus F1 animals are necropsied and examined for malformations after determination of the age of puberty. After termination of the breeding phase of the study, the F1 rats are necropsied, as were the P0 generation rats.

In these multigenerational studies, we did not examine some of the variables that we now know to be exquisitely sensitive to antiandrogenic action (AGD, areolas, or nipples). In this regard, the shorter-term transgenerational studies were designed to determine if administration of L or DBP during sexual differentiation exhibited antiandrogenic activity at dosage levels that are not overtly toxic to the animals (Khera and Ruddick, 1973; Ema et al., 1994).

Transgenerational Studies (In Utero or Perinatal Dosing)
For the transgenerational toxicology studies, dams were dosed only with the chemicals during gestation (starting after implantation or later during fetal sexual differentiation) and lactation. The offspring and the sire were not exposed to the chemicals directly. Typically, pup body weight and AGD were measured at postnatal day (PND) 2 and at 10–13 days of age, and offsprings were examined (in a blinded manner) for areolas (dark focal areas lacking hair in the appropriate anatomical region are scored as areolas) with and without a nipple bud. The examination of 'nipples' in these experiments typically used a dissecting scope to confirm the presence of a 'bud' within the areola. At 28 days of age, male pups were weaned and housed in groups of two or three with litter mates, and from 35 days on, each male was examined for preputial separation (an index of puberty). After puberty, males were examined for external malformations, and during adulthood, the animals were necropsied and reproductive tissues evaluated.

Transgenerational Experiment One: Linuron, DBP, DEHP, Chlozolinate, and Iprodione
Linuron (lot no. 225, 100% technical grade) was acquired from Dupont Chemical. Laboratory grade corn oil was the vehicle, with L (in 2.5 μl of corn oil [g body weight]$^{-1}$) being administered by gavage at 0 or 100 mg kg^{-1} day^{-1} from day 14 to 18 of pregnancy. DBP (lot no. 109F0386, 99.8% purity) and DEHP (lot no. 106H3487, > 99% purity) were both obtained from Sigma Chemical. DBP (500 mg kg^{-1} day^{-1}) was given during pregnancy day 16–19 in one study, while DBP (500 mg kg^{-1} day^{-1}) and DEHP (750 mg kg^{-1} day^{-1}) were given from day 14 of pregnancy to day 3 of lactation in another study. The two dicarboximide fungicides, chlozolinate (lot no. 05072, 98% purity) and iprodione (lot no. 30500, > 99% purity), were both purchased from Riedel-de Haën and were administered at 100 mg kg^{-1} day^{-1} from GD 14 to day 3 of lactation. All the doses administered included daily adjustments based on individual maternal weight changes throughout the dosing period.

This first study used 50 pregnant SD dams, being randomly assigned (in a manner that provided equal means and distributions in maternal weight) to one of six treatment groups (control, $n = 10$; L, $n = 8$; DBP, $n = 8$; DEHP, $n = 8$; chlozolinate, $n = 8$; and iprodione, $n = 8$). At about 5 months of age, males were killed by decapitation within 15 s of removal from the home cage in a separate room during the dark phase of the animal's daily cycle. The ventral surface of each male was shaved and examined for abnormalities, including the number and location of retained nipples, cleft phallus, vaginal pouch, and hypospadias. The animals were examined internally for ectopic or atrophic testes; agenesis of the gubernaculum, epididymides, sex accessory glands, and ventral prostate; epididymal granulomas; hydronephrosis; and enlarged bladder with stones. Weights measured included body, pituitary, adrenal, kidney, liver, ventral prostate, seminal vesicle (with coagulating gland and fluid), testis, and epididymis. Testicular, epididymal, and sex accessory tissues were taken for ongoing histological evaluation, and,

in some cases, tissue was taken to confirm the presence of retained nipples in male offspring. Tissues were placed in Bouin's fixative for 24 h, after which they were rinsed and stored in 70% alcohol, embedded in paraffin, stained with hematoxylin and eosin, and were sent to Experimental Pathology Laboratories (Research Triangle Park, NC) for examination of histopathological lesions. In a second study, conducted as above with LE Hooded rats, DBP or the vehicle were administered by gavage at 500 mg kg^{-1} day^{-1} from GD 16–19 with five treated and seven control litters (all males per litter were examined).

Transgenerational Experiment Two: Procymidone and p,p'-DDE

Pregnant LE hooded rats were treated with either P at 100 mg kg^{-1} day^{-1} from GD 14 to PND 3 (Riedel-de Haën, lot no. 72480, 99% purity, in laboratory grade corn oil, Sigma Chemical), p,p'-DDE at 100 mg kg^{-1} day^{-1} from GD 14–18 (Janssen Pharmaceutical, lot no. 10230AZ, > 99% purity), or the vehicle ($n = 8$/group). Procymidone was administered at a suspension that was stirred continuously while the rats were being dosed. Offsprings were examined as described earlier. Males were necropsied at about 15 months of age to attempt to replicate the observed inflammation of the prostate seen by Ostby et al. (this issue) in middle-aged males. Selected reproductive tissues (ventral prostate and seminal vesicles) were placed in Bouin's fixative for 24 h, after which they were rinsed and stored in 70% alcohol, embedded in paraffin, stained with hematoxylin and eosin, and were sent to Experimental Pathology Laboratories, Inc. (Research Triangle Park, NC) for examination of histopathological lesions. We also repeated the p,p'-DDE study with SD rats ($n = 9$ controls and 11 treated dams). SD male offspring were necropsied at about 10 months of age.

Transgenerational Experiment Three: PCB 169

Pregnant LE hooded rats (nine dams per group) were treated once by gavage with PCB 169 (Crescent Chemical, lot no. 940214, 99.5% purity) at 1.8 mg kg^{-1} on GD 8. This dosing regime is similar to that used by Smits-van Prooije et al. (1993) and exposes the developing embryos and fetuses over the entire period of gestation due to the long half-life of this congener in the rat. In addition, we have found (Gray and Ostby, 1995; Gray et al., 1995a) that dosing at this stage of pregnancy with TCDD alters reproductive development in both male and female LE hooded rats. In this experiment, AGD was measured on 3-day-old pups, whereas our other studies examined this endpoint at day 2, and males were necropsied at 18–20 months of age. Selected reproductive tissues (epididymis, dorsolateral prostate, and ventral prostate) were placed in Bouin's fixative for 24 h, after which they were rinsed and stored in 70% alcohol, embedded in paraffin, stained with hematoxylin and eosin, and were sent to Experimental Pathology Laboratories, Inc. (Research Triangle Park, NC) for examination of histopathological lesions.

Transgenerational Experiment Four: Ketoconazole

A study was conducted to determine if this compound demasculinized male LE hooded rat offspring as a consequence of the inhibition of fetal male T synthesis. Pregnant LE hooded dams (seven to eight per group) were dosed with ketoconazole at 0, 12.5, 25, and 50 mg kg^{-1} day^{-1} from day 14 of pregnancy to day 3 of lactation. Previously, it was determined that administration of ketoconazole at 100 mg kg^{-1} day^{-1} resulted in interruption of pregnancy in all treated females within a few days. In this experiment, male offspring were necropsied at about 5 months of age. Due to the lack of effect of ketoconazole on any variable measured in this experiment, reproductive tissues were not examined for histological alterations.

Transgenerational Experiment Five: EDS

Ethane dimethane sulphonate specifically kills Leydig cells in adult male rats when administered by injection. It has been reported that oral treatment is effective as well (Jackson, 1973). In addition, several publications have demonstrated that EDS destroys fetal populations of Leydig cells postnatally (Morris et al., 1988a; Zaidi et al., 1988). The current experiment was designed to determine if oral perinatal maternal treatment with EDS would demasculinize male rat offspring. In this regard, there were five controls and five LE hooded rats that were treated with EDS at 50 mg kg^{-1} day^{-1} (in water) from day 14 to 21 of pregnancy. Treatment was terminated at day 21 of gestation because the EDS-treated dams not only failed to gain weight normally, their body weight was reduced as compared to GD 14 body weight.

Uterotropic Study To Determine the Estrogenic Potential of DBP and Other Xenobiotics

Although some investigators have suggested that DBP (Jobling et al., 1995) is estrogenic *in vitro*, this endocrine activity has not been confirmed *in vivo*. In this experiment, we exposed adult ovariectomized female rats to estradiol benzoate (10 μg, s.c.) and DBP (200 and 400 mg kg^{-1} day^{-1}, s.c. and 1000 mg kg^{-1} day^{-1} orally). For comparison, several known xenoestrogens, octylphenol (200 mg kg^{-1} day^{-1}, s.c.), bisphenol A (200 mg kg^{-1} day^{-1}, s.c.), and methoxychlor (200 and 400 mg kg^{-1} day^{-1}, s.c. and orally), were also tested for estrogenic activity. In this 3 day assay, females were injected s.c. with the treatments on the morning of the first 2 days, which was followed by 0.5 mg progesterone s.c. on morning of the third day. In this protocol (Gray and Ostby, 1998), estrogenic compounds induce a uterotropic response and lordosis behavior (when paired with a sexually active stud male rat during

the dark phase of the animals activity cycle 6–8 h after progesterone injection). For lordosis behavior, the ratio of lordosis responses to mounting was calculated (termed the lordosis quotient). Following the behavioral observation, females were euthanized with CO_2 and necropsied, and the uterus was trimmed of mesenteric fat and weighed with and without lumenal fluid. The study was conducted in a series of incomplete blocks with six animals in most treatment groups. As each block contained both an estradiol-positive control and a vehicle (corn oil)-injected control, these two groups have a larger sample size.

Statistical Analyses

For multigenerational studies of L and DBP, parametric data were analyzed using a one-way analysis of variance (ANOVA) model on General Linear Models Procedure (PROC GLM) from the Statistical Analysis System (SAS), available on the EPA IBM mainframe computer. For statistical purposes, analysis of P0 data used the numbers of individuals as the experimental unit, while data from the F1 offspring used the numbers of litters, not the numbers of pups, as the experimental unit. For the transgenerational studies, analyses of F1 offspring data also used the numbers of litters as the experimental unit. Hence, AGD, growth, pubertal, and necropsy data were analyzed by ANOVA on PROC GLM using litter mean values rather than individual values. When the overall ANOVA was significant ($p < 0.05$), then LSMEANS were used to compare individual treatments to the control group (a two-tailed *t*-test, appropriate for a priori hypotheses based on known effects of antiandrogenic chemicals or PCBs on sexual differentiation). Categorical data were analyzed using Fisher's exact test. For the uterotropic study, data were analyzed after log transformation to correct for heterogeneity of variance using an ANOVA model on PROC GLM. When the overall ANOVA was significant ($p < 0.05$), then LSMEANS (appropriate for a priori hypotheses based on the expectation that a xenoestrogen will increase uterine weight and the display of lordosis behavior) was used to compare individual treatments to the control group.

Linuron result:

In the multigenerational study, subchronic L administration from weaning through puberty, breeding, and lactation in LE hooded rats was without significant effect on any serum hormone concentration in the P0 generation (data not shown). Linuron treatment did not induce endocrine changes that are typically seen with anti-androgens like flutamide and V, which produce elevated serum LH and T levels. However, some reproductive alterations were detected in our study that could have been mediated by antagonism of androgen-AR function. Seminal vesicle and cauda epididymal weights (Figure 1) and the age at puberty (Figure 2) were reduced or delayed in P0 male rats (directly exposed by gavage), all of which are androgen-dependent variables, by treatment with L at 40 mg kg^{-1}

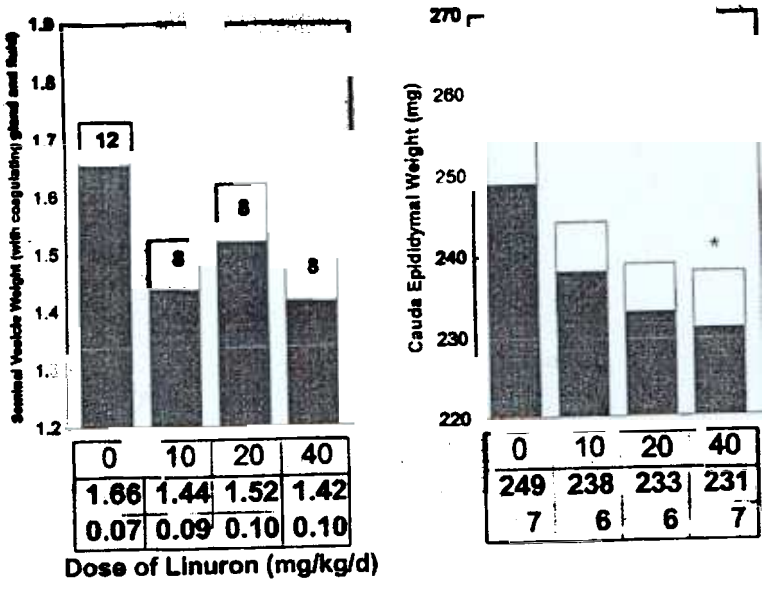


Figure 1. When linuron was administered orally in oil at 0, 10, 20, or 40 mg kg^{-1} day^{-1} for 80 days starting at weaning, treatment did not alter body, liver, or paired adrenal weights. Seminal vesicle and cauda epididymal weights were significantly reduced ($p < 0.05$ by a *t*-test, but not ANOVA) in male LE hooded rats. Cauda epididymal sperm numbers were reduced, but this effect was not statistically significant. The white stacked bars represent the SE of the means. The actual means and SEs also are displayed in the second and third rows of the table below the figure. The dose group is given in the top row of the table. The numbers in the SE bar are the numbers of animals in each group.

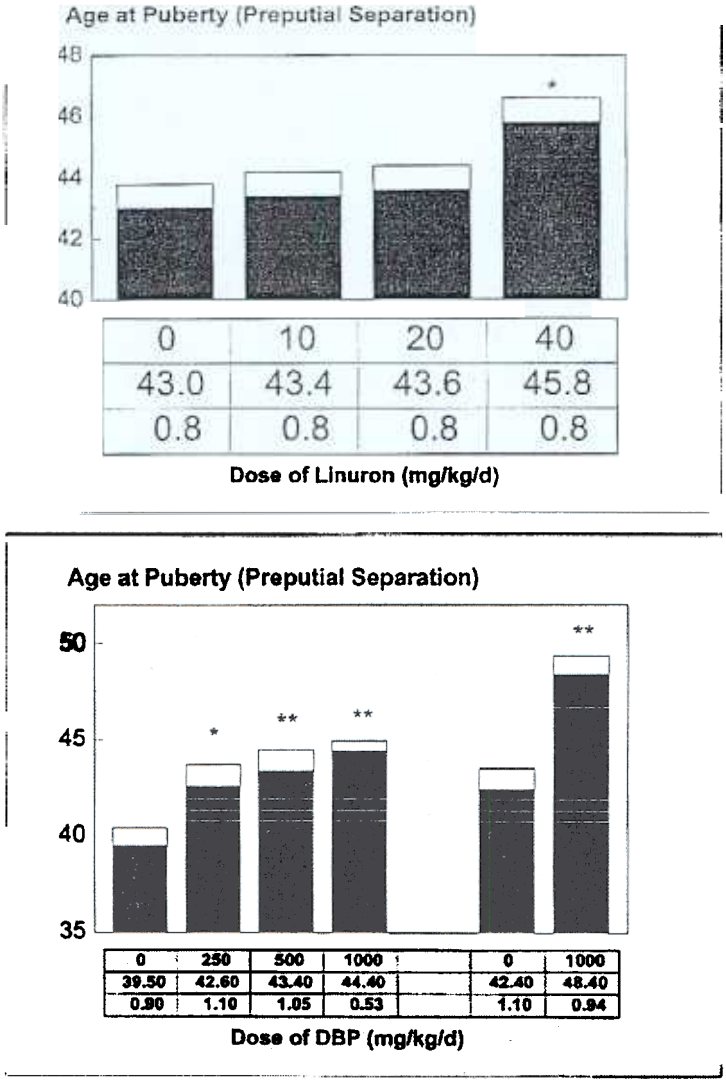


Figure 2. Oral linuron treatment for 80 days, starting at weaning (0, 10, 20, or 40 mg kg^{-1} day^{-1}), delayed preputial separation in LE hooded male rats, an androgen-dependent index of puberty (top panel). In addition, oral dibutyl phthalate (DBP) treatment also delayed puberty. DBP data are from two studies. The white stacked bars represent the SE of the means. The actual means and SEs also are displayed in the second and third rows of the table below the figure. The dose group is given in the top row of the table. * indicates $p < 0.05$; * * indicates $p < 0.01$.

day^{-1}. In addition to these subtle reproductive effects, seen in the P0 generation, F1 rats, exposed to L *in utero* and via milk during lactation, produced fewer pups (65 *versus* 104, $p < 0.01$) when mated continuously over 12 breeding cycles (Figure 3), and the male offspring displayed reduced testes weight (1.40 g $\pm$ 0.10 *versus* 1.74 g $\pm$ 0.045, $p < 0.004$), spermatid numbers (118 million $\pm$ 12 *versus* 140 $\pm$ 6.2, $p < 0.12$), and epididymal weight (490 mg *versus* 601 mg, $p < 0.0005$). Such developmental reproductive effects are surprising because it has been reported that (1) L is not teratogenic (Khera et al., 1978); and (2) there was no indication that the administration of L in the diet over three generations produced malformations of the reproductive tract of the male offspring (Hodge et al., 1968).

In the transgenerational study, administration of L at 100 mg kg^{-1} day^{-1} reduced maternal weight gain in SD rats by 35 g during the treatment period. However, L did not cause maternal death, and weight gain from day 18, when dosing was terminated, until day 22 was near normal (44 g in control *versus* 35 g for the L dams). Pup weight at birth was reduced by 20% by L treatment ($p < 0.0001$), and AGD in male offspring, adjusted by analysis of covariance

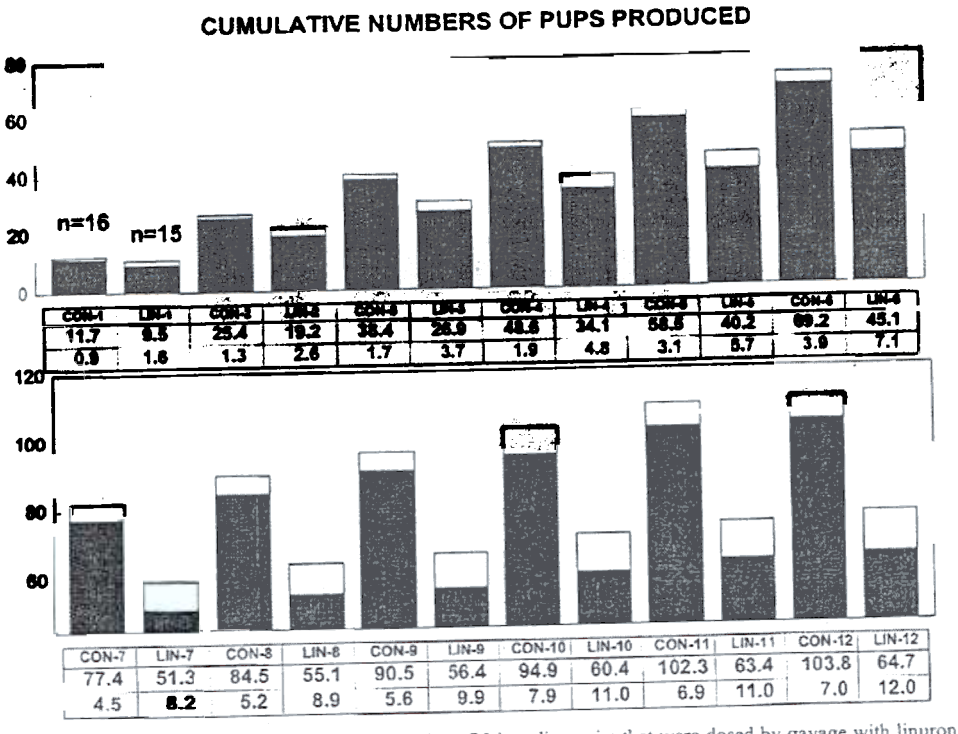


Figure 3. Male and female LE hooded rat offspring (F1) were derived from P0 breeding pairs that were dosed by gavage with linuron in corn oil at 40 mg kg^{-1} day^{-1} from weaning through puberty, mating, gestation, and lactation. Treated F1 rats produced significantly fewer pups when mated under continuous breeding conditions over 12 breeding cycles. In this study, the F1 only received transplacental and lactational linuron exposure. The white stacked bars represent the SE of the means. The dose group is given in the top row of the table, while means and SEs also are displayed in the second and third rows of the table below the figures. The overall fecundity of the linuron is significantly reduced compared to control pairs. Con-1 refers to the first control litter, while Lin-1 refers to the first treated litter, etc. The *n* values in the top panel are the numbers of F1 breeding pairs used in this study.

for body weight, was also significantly ($p < 0.005$) reduced by about 30% (Table 1). The reduction in AGD produced by L treatment is considerably larger than the 15% reduction in AGD seen in male pups whose body weight was reduced by 45% as a consequence of EDS treatment. In addition, the incidence of areolas (with and without nipples) seen in the male offspring as infants was increased from 0% in controls to more than 44% in the L-treated males, reflecting the inhibition of DHT-induced regression of nipple anlagen *in utero* by L. The reduction in body weight may be endocrine-mediated. Linuron has been shown to reduce serum thyroxine and hypothalamic neuroendocrine levels in adult males treated for 4 days at this dosage level (Rehnberg et al., 1988).

At necropsy, one male out of 13 from the L group displayed epispadias (partial hypospadias with the urethral opening being half way down the phallus). Numerous epididymal and testicular malformations were noted in the L group, with more than 50% of the males displaying agenesis or atrophy of one or both organs. Epididymal malformations included agenesis of the caput and/or corpus epididymides, while some testes were atrophic, fluid-filled, and flaccid. However, males in the L group did not display testicular agenesis. Androgen-dependent tissues were permanently reduced in size, including the seminal vesicles, ventral prostate, levator ani/bulbocavernosus muscles, and the epididymides (using weights of tissues that did not display agenesis), and testes weights also were reduced (excluding data from animals that display severe atrophy) (Table 1).

Phthalate (DBP and DEHP) results

In the 3-day uterotropic and sex behavior (lordosis) assay, with treatments administered for 2 days and followed on the third day by 0.5 mg progesterone s.c., DBP treatment (s.c. at 200 or 400 mg kg^{-1} day^{-1} or by gavage at 1000 mg kg^{-1} day^{-1}) failed to induce estrogenic responses in either the uterus or the brain (Figure 4). In contrast, the positive control estradiol (estradiol benzoate, s.c. at 10 μg rat^{-1} day^{-1}) and the known xenoestrogens used here (bisphenol A, octylphenol, and methoxychlor) all induced positive uterine and behavioral responses. The lordosis quotient (LQ) is always zero in ovariectomized controls

Table 1. *In utero* or perinatal exposure to either dibutyl phthalate (DBP), diethylhexyl phthalate (DEHP), or linuron alters reproductive development in male rat offspring[a]

Chemical	Control	DBP (500 mg kg^{-1} day^{-1})	DEHP (750 mg kg^{-1} day^{-1})	Linuron (100 mg kg^{-1} day^{-1})	Control	DBP[b] (500 mg kg^{-1} day^{-1})
Strain/number of litters/ number of males	SD/9/50				LE/6/19	
Days treated		GD14–PND3	GD14–PND3	GD14–GD18		GD16–19
Pup weight (g) day 2	7.6 ± 0.19	7.1 ± 0.25	6.3 ± 0.23 $p < 0.0002$	6.0 ± 0.19 $p < 0.0001$	6.92 ± 0.27	6.60 ± 0.34
Anogenital distance (mm) day 2 (with ANCOVA-bw)	3.70 ± 0.09	2.79 ± 0.09 $p < 0.0001$	2.45 ± 0.11 $p < 0.0001$	2.57 ± 0.10 $p < 0.005$	3.21 ± 0.07	2.83 ± 0.05 $p < 0.0008$
Percentage of areolas	0%	55% ± 14	88% ± 12	44% ± 15	0/0	87% ± 0.12
Number of areolas	0	2.7 ± 0.75	8.4 ± 15	2.1 ± 0.7		4.1 ± 1.0
Percentage of hypospadias	0	6.2 ± 6.2	67 ± 14	12.5 ± 12.5	0	0
Percentage of vaginal pouch	0	0	45 ± 17	0	0	0
Percentage of ventral prostate agenesis	0	0	14 ± 14	0	0	0
Percentage of testicular and epididymal atrophy or agenesis	0	45.8 ± 12	90 ± 10	56 ± 18	0	0
Number retained nipples	0	2.2 ± 0.8 $p < 0.0003$	8.1 ± 1.4 $p < 0.0001$	2.1 ± 0.7 $p < 0.002$	0	.9 ± 1.6 $p < 0.032$
Adult male rat offspring necropsy data						
Body weight (g) (litter/males)	566 ± 14 (9/25)	563 ± 13 (8/17)	546 ± 17 (7/17)	503 ± 25 (8/13) $p < 0.01$	632 ± 42 (6/16)	654 ± 55 (4/13)
Seminal vesicle (mg)	1826 ± 52	1563 ± 95	638 ± 235 $p < 0.0001$	1606 ± 84	2019 ± 92	1612 ± 163 $p < 0.02$
Ventral prostate (mg)	704 ± 23	525 ± 34 $p < 0.03$	386 ± 90 $p < 0.0004$	460 ± 56 $p < 0.004$	465 ± 51	370 ± 64 $p < 0.30$
Epididymides (mg)	1348 ± 38	1060 ± 99 $p < 0.015$	890 ± 264 $p < 0.015$	957 ± 160 $p < 0.003$	1325 ± 23	1311 ± 56
Cauda epididymis (mg)	325 ± 7	219 ± 28 $p < 0.005$	155 ± 53 $p < 0.005$	211 ± 55 $p < 0.004$	322 ± 7	307 ± 21
Testes (mg)	3607 ± 77	3055 ± 268 $p < 0.03$	2368 ± 353 $p < 0.0001$	2815 ± 184 $p < 0.004$	3762 ± 110	3923 ± 130
Glans penis (mg)	112 ± 1.5	93 ± 2.4 $p < 0.0001$	71 ± 3.8 $p < 0.0001$	94 ± 3.3 $p < 0.0002$	122 ± 3.4	122 ± 5.3
Number of nipples	0	2.2 ± 0.8 $p < 0.03$	8.1 ± 1.4 $p < 0.0001$	2.1 ± 0.7 $p < 0.03$	0	1.9 ± 1.6 $p < 0.03$
Levator ani-bulbocavernosus (mg)	1254 ± 50	941 ± 31 $p < 0.0001$	671 ± 67 $p < 0.0001$	1053 ± 21 $p < 0.003$	1287 ± 43	1076 ± 79 $p < 0.03$

[a]In the first study, male offspring dams were necropsied at about 6 months. In the second study, male offspring were necropsied at 9 months. Data analyzed by litter means rather than individual values.

[b]DBP treatment on days 12–15 was much less effective (data not shown).

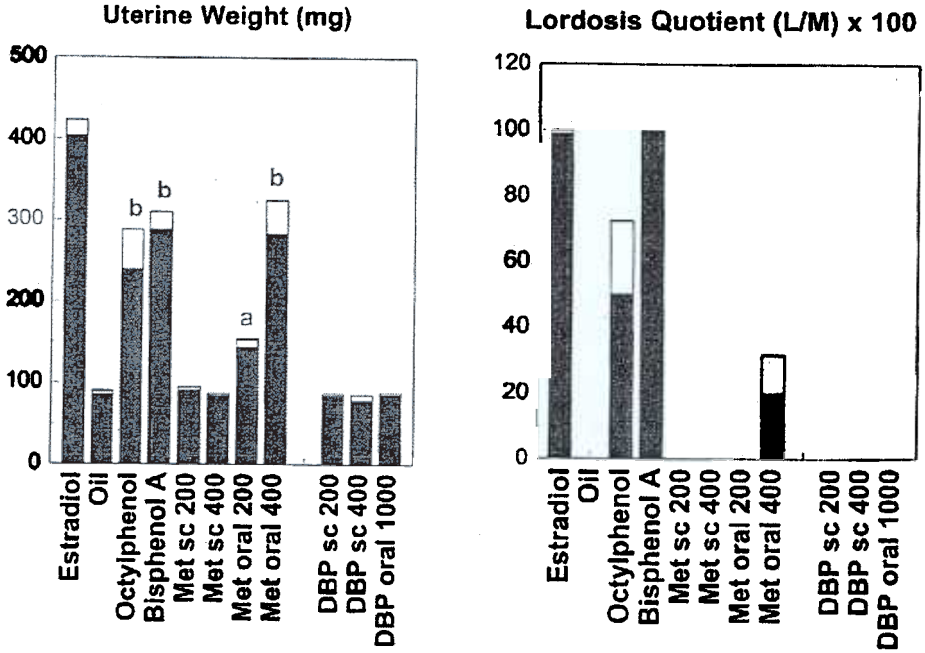


Figure 4. In contrast to dibutyl phthalate (DBP), xenoestrogen treatments (bisphenol A, octylphenol, and oral but not s.c. methoxychlor) stimulated an increase in uterine weight and induced estrogen-dependent sex behavior (lordosis). In this study, female SD rats were ovariectomized for at least 4 weeks, treated for 2 days with a chemical (n = at least 6 per group), injected with 0.5 mg progesterone on the morning of the third day, and examined for the display of lordosis behavior elicited by the mounting of a stud male (in the afternoon after lights out). The lordosis quotient (LQ) = the percentage of times that the females displayed lordosis behavior after being mounted. LQ is typically determined by examining 5–10 mounts. After observation of lordosis behavior, females were necropsied and uterine weight (shown here with fluid) was measured. In this figure, all values greater than 100 mg are statistically significant. Lordosis behavior is never displayed by females that are not exposed to an estrogenic chemical. Estradiol, octylphenol (200 mg kg^{-1} day^{-1}, s.c.), bisphenol A (200 mg kg^{-1} day^{-1}, s.c.), and methoxychlor (oral at 200 and 400 mg kg^{-1} day^{-1}) increased uterine weight. All of these treatments, with the exception of methoxychlor at 200 mg kg^{-1} day^{-1}, also induced lordosis behavior. DBP (s.c. 200 and 400 mg kg^{-1} day^{-1} or oral at 1000 mg kg^{-1} day^{-1}) did not increase uterine weight or induce lordosis behavior. 'a' indicates $p < 0.05$, while 'b' refers to $p < 0.01$.

that have not been estrogen treated. Interestingly, s.c. methoxychlor treatment at 200 and 400 mg kg^{-1} day^{-1} was negative for both measures in this assay while, in contrast, oral treatment at 400 mg kg^{-1} day^{-1} was effective.

In our multigenerational assessment of the reproductive effects of DBP using the LE hooded rats, daily administration of 250, 500, and 1000 (males only at this dose) mg kg^{-1} day^{-1} in the P0 generation significantly (p at least < 0.05) delayed puberty at all dosage levels (Figure 2). DBP treatment did not accelerate the age at vaginal opening (pseudoprecocious puberty) or induce persistent vaginal cornification (data not shown), effects that are indicative of subchronic estrogen exposure (Gray et al., 1989, 1997c; Gray and Ostby, 1998). In the P0 generations, dosage levels of 500 and 1000 mg DBP kg^{-1} day^{-1} significantly ($p < 0.01$) reduced fertility in both male and female rats (mated to untreated animals). Infertility in P0 male rats was related to testicular atrophy and reduced sperm production, while treated females cycled and mated successfully, but many DBP-treated females (at 500 mg kg^{-1} day^{-1}) aborted their litters around midpregnancy.

In the F1 offspring, *in utero* and lactational exposure via the dam to 250 and 500 mg DBP kg^{-1} day^{-1} induced reproductive and nonreproductive malformations and reduced fecundity of the LE hooded rat offspring (in similarly treated mating pairs under continuous breeding conditions) (Figure 5). Malformations included a low incidence of hypospadias, testicular nondescent, anophthalmia, uterus unicornous, and renal agenesis (data not shown). In addition, F1-treated males exhibited reduced cauda epididymal sperm numbers (Figure 5). In the F1, the LOAEL (lowest-observed-adverse-effect level) was 250 mg kg^{-1} day^{-1}, but a NOAEL was not established.

In the first transgenerational study using DBP, short-term dosing (4 days) with 500 mg DBP kg^{-1} day^{-1} on GD 16–19 reduced AGD in LE hooded male progeny, induced retained nipples, and permanently reduced androgen-dependent tissue weights (Table 1).

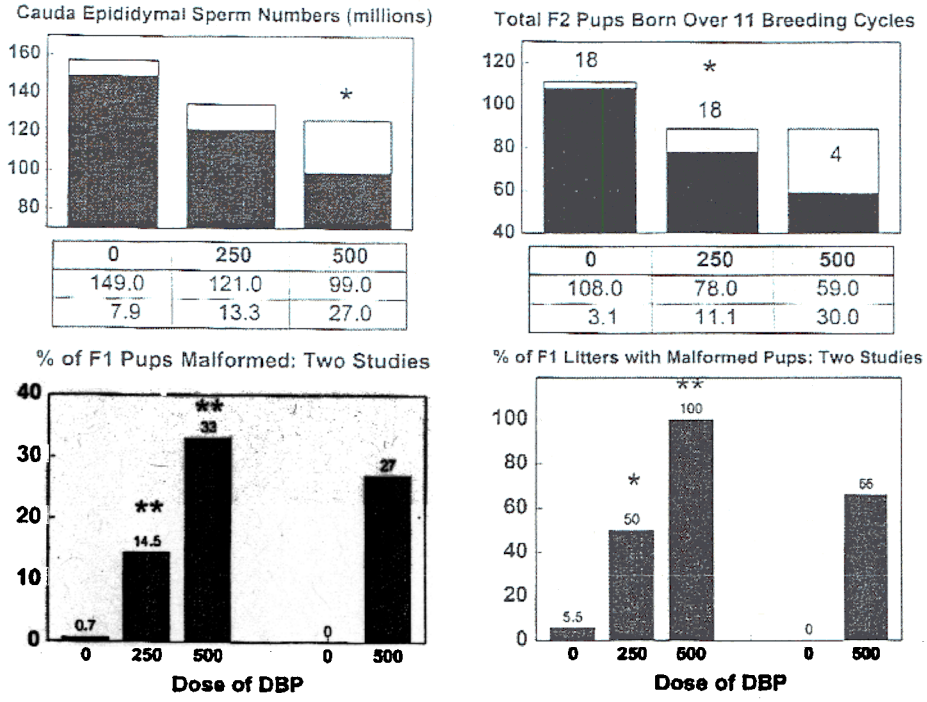


Figure 5. The LE hooded rats were treated by gavage with dibutyl phthalate (DBP) in oil at 250 or 500 mg kg^{-1} day^{-1} from weaning through puberty, mating, gestation, and lactation (P0 generation). Their offspring, the F1, produced significantly fewer pups when mated under continuous breeding conditions over 11 breeding cycles. In addition, F1-treated males exhibited reduced cauda epididymal sperm numbers. Treated F1 male and female rats also displayed urogenital malformations/abnormalities including a low incidence of agenesis of the epididymis, hypospadias, ectopic testis, renal agenesis, and uterine abnormalities (partial agenesis or lack of implants in one uterine horn). In addition, a few treated animals displayed anophthalmia. In these two studies, the F1 only received transplacental and lactational DBP exposure. The number of pups/litters is 179/24, 76/10, and 20/4 for the control, 250, and 500 mg DBP kg^{-1} day^{-1}, respectively. An * indicates that $p < 0.05$; ** indicates $p < 0.01$. For malformation data, studies were pooled for statistical analysis (by chi-square for the pup and Fisher's exact for the litter incidence data). The numbers above the bars in the top right panel are the numbers of F1 breeding pairs in this study. The white stacked bars represent the SE of the means. The dose group is given in the top row of the table, while means and SEs also are displayed in the second and third rows of the table below the figures.

In another transgenerational study, we compared the developmental reproductive toxicity of DBP to DEHP in the SD rat. This study was part of the preceding experiment using L. In this portion of the experiment, dams were dosed by gavage from GD 14 to lactational day 3 with DBP at 500 mg kg^{-1} day^{-1} or DEHP (in corn oil) at 750 mg kg^{-1} day^{-1} (approximately equimolar to 500 mg DBP kg^{-1} day^{-1}). DBP altered sexual differentiation in both SD and LE hooded rats. The effects were more pronounced in SD rats exposed for a longer period (12 days) than in LE rats (4 days). DEHP was considerably more toxic than was DBP to the reproductive system of the male offspring (Table 1). DEHP induced high levels of testicular and epididymal abnormalities, including atrophy and agenesis. A striking effect of DEHP was noted in 8-day old pups (Figure 6). Several males from different litters displayed hemorrhagic testes that were visible by gross examination of the inguinal region. Obviously, the testis is a direct target of DEHP during perinatal life. Testicular alterations of this nature have not been observed with other 'antiandrogens,' which indicates that the phthalate affects the developing reproductive system by a mechanism that is distinct from previously described developmental reproductive toxicants.

Chlozolinate and iprodione results

Chlozolinate and iprodione did not demasculinize or feminize male pups after exposure to 100 mg kg^{-1} day^{-1} from GD 14 to PND 3 (data not shown).

Procymidone results

The P treatment demasculinized and feminized the male rat offsprings in a manner nearly identical to that seen with V (Gray et al., 1994). P-treated male offsprings displayed reduced AGD, areolas, permanent nipples, reduced sex accessory gland size, and hypospadias with a vaginal pouch (Table 2). In this experiment, P was more potent than *p,p'*-DDE with respect to the degree of reduction in

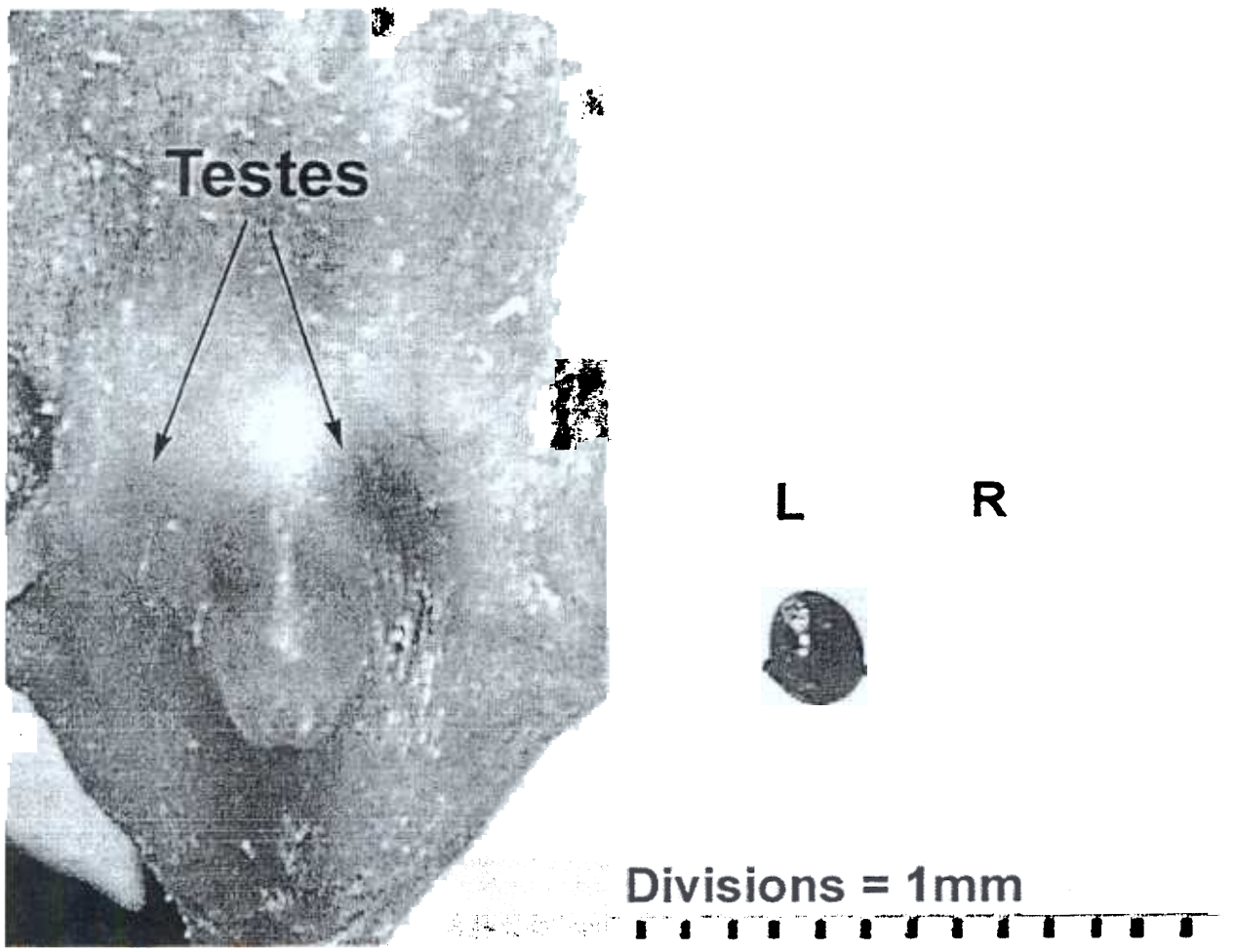


Figure 6. Maternal treatment with diethylhexyl phthalate (DEHP) at 750 mg kg^{-1} day^{-1} from gestational day 14 to lactational day 3 produces testicular abnormalities in the offspring. This 8-day-old infant Sprague–Dawley male rat displayed discoloration of the left testis that was visible through the skin. When necropsied, it was evident that the testis and the epididymides were hemorrhagic. This unusual effect was seen in several other males from many litters. The F1 only received transplacental and lactational DEHP exposure.

AGD and sex accessory gland size and the induction of hypospadias (Table 2). In addition, P induced chronic active ventral prostatitis and vesiculitis, while *p,p'*-DDE did not. Mononuclear cell infiltration or the more severe chronic-active prostatitis (ventral lobe) was present in 47% (9/19) of the P group, with 36% (6/19) having chronic-active vesiculitis. This is nearly identical to the 42% incidence of histological lesions of the ventral prostate of similarly exposed old male offspring from the 100 mg P kg^{-1} day^{-1} dosage group in our dose response study with P (Ostby et al., this issue). Several nipple buds were histologically confirmed in the P group as well.

p,p'-DDE results

The *p,p'*-DDE treatment was limited to GD 14–18 because it reduced maternal weight gain by about 35 g during treatment. Maternal weight gain was normal in dams after *p,p'*-DDE administration was terminated, and pup weight at 2 days of age was not significantly reduced (for the SD rat, controls = 6.92 ± 0.16 g *versus* DDE treated = 6.67 ± 0.10 g). SD male rats appeared to be more affected than did LE hooded males (Table 2). SD rats displayed hypospadias and increased numbers of retained nipples as compared to treated LE rats. It is uncertain if this reflects a true strain difference in sensitivity or if it merely results from experiment to experiment variation. However, this is the third experiment in which we exposed LE rats and we have yet to see hypospadias in *p,p'*-DDE-treated male LE rat offspring. You et al. (1998) have studied the effects of *p,p'*-DDE on the male offspring using the same protocol as that used here. They also found that *p,p'*-DDE induced antiandrogenic effects on AGD and areola development in both LE and SD rat strains.

Table 2. Maternal administration of either 100 mg kg^{-1} day^{-1} p,p'-DDE on days 14–18 of gestation or procymidone from day 14 of gestation to 3 days of age alters reproductive development of male rat offspring

	Long–Evans hooded			Sprague–Dawley	
	Control	DDE	Procymidone		DDE
Number of litters (number of males necropsied)					
Anogenital distance (mm) (no males)	2.85 ± 0.04 (61)	2.69 ± 0.06 (42)	2.17 ± 0.08[a] (54)	2.76 ± 0.10 (49)	2.51 ± 0.08[a] (83)
Body weight (g) day 2	7.08 ± 0.12	6.75 ± 0.21	6.72 ± 0.11	6.92 ± 0.16	6.67 ± 0.10
Percent with areolas	0	21 ± 10[a]	100[a]	0	71 ± 9[a]
Mean number of retained nipples	0	0.74 ± 0.15[a]	3.75 ± 0.83[a]	0	3.13 ± 0.5[a]
Adult body weight (g)	737 ± 28	710 ± 22	681 ± 27	638 ± 8	613 ± 9
Glans penis (mg)	no data	–	–	112 ± 2.9	102 ± 1.5[a]
Cauda epididymis (mg)	no data	–	–	331 ± 9.6	305 ± 6.2[a]
Testes (g)	3.79 ± 0.10	3.86 ± 0.08	3.99 ± 0.12	3.40 ± 0.08	3.42 ± 0.07
Ventral prostate (MG)	529 ± 25	417 ± 23[a]	233 ± 44[a]	747 ± 36	575 ± 29[a]
Seminal vesicle (mg)	1744 ± 74	1774 ± 55	1334 ± 204[a]	2015 ± 11	1931 ± 51
Levator ani-bulbocavernosus muscles (mg)	no data	–	–	1400 ± 40	1204 ± 23[a]
Percent with hypospadias	0	0	40[a]	0	7.8 ± 7.8
Epididymides (paired) (mg)	1357 ± 30	1382 ± 29	1353 ± 30	1328 ± 36	1260 ± 24
(1) Atrophy, (2) chronic active prostatitis or focal mononuclear cell infiltration	(1) 0/26, (2) 1/26	(1) 8/27[a], (2) 0/27	(1) 5/19, (2) 9/19[a]	no data	no data
Chronic-active vesiculitis	0/26	0/27	6/19[a]	no data	no data

[a]Differs significantly from control values.

Table 3. The effects of a single dose (1.8 mg kg^{-1}) of PCB 169, an Ah receptor agonist, on gestational day 8 on reproductive development in LE hooded rat offspring[a]

	Treatment Group	
	Control	PCB 169 (1.8 mg kg^{-1} GD 8)
Number of litters		9
Incidence of maternal or neonatal death		0
Male body weight (g) at day 3		8.03 ± 0.21
Female body weight (g) at day 3		7.67 ± 0.22
Male anogenital distance at day 3 (mm)		3.44 ± 0.06
Female anogenital distance at day 3 (mm)		1.79 ± 0.04
Incidence of areolas (with or without nipples) in infant males		0
Litter mean percent eye opening: at 14 days of age		17 ± 0.05 ($p < 0.03$)*[c]
At 15 days of age		83 ± 0.07 ($p < 0.0002$)*
At 16 days of age		100 ± 0 ($p < 0.04$)*
Age at puberty in males (days)		50.2 ± 3.1 ($p < 0.04$)*
Weight at puberty in males (g)		227 ± 9.3
Age at puberty in females (days)		30.3 ± 0.15
Weight at puberty in females (g)		93.6 ± 6.6
Percentage of females with a transient vaginal thread		72.3 ± 15*
Percentage of females with a permanent vaginal thread		55.6 ± 13.9*
Female necropsy data[b]		
Percentage of those with mild hypospadias	0	57 *
Percentage of those with cleft phallus	0	36 *
Size of the urethral slit (mm)	1.37 ± 0.08	2.87 ± 0.24
Distance from urethral to vaginal opening (mm)	10.27 ± 0.30	8.37 ± 0.38
Percentage of those with ovarian atrophy	0	14
Ovarian weight	100.8 ± 9.4	89.8 ± 11.5

[a]PCB 169 treatment did not affect maternal or neonatal body size or viability (data not shown).
[b]Prenatal PCB 169 treatment alters the external genitalia, measured in 70-day old female offspring. Body, brain, liver, kidney, adrenal, and pituitary weights were unaffected (data not shown).
[c]Indicates that the value differs significantly from control value by at least $p < 0.05$.

Putative antiandrogenic effects of PCB 169, an Ah receptor agonist

The profile of effects seen in PCB 169-treated offspring is similar to that produced by exposure to TCDD at 1 μg kg^{-1} on day 15 of pregnancy. However, the effects in the PCB 169 male are more pronounced than what we saw in our TCDD-treated animals. Similar to TCDD treatment, PCB 169 treatment accelerated the age at eye opening and induced vaginal threads and mild hypospadias (urethral opening separate from the vaginal canal with cleft phallus) in female offspring, without reducing AGD or inducing areolas or nipples in males (Table 3). In TCDD-treated males, reductions in ventral prostate, seminal vesicle, and testis size are displayed during peripubertal life (49–63 days of age) but they are attenuated with age (Gray et al., 1997a). In this regard, these organs were more affected in PCB 169-treated males at 65 days of age than in middle-aged males (Table 4). This general delay in development of all reproductive events during puberty (sex accessory growth, onset of spermatogenesis, preputial separation) raises the possibility that these effects arise from altered hypothalamic–pituitary maturation rather than from direct effects of PCB on the reproductive tract tissues themselves. As compared to males exposed on GD 8 to 1 μg TCDD kg^{-1}, PCB 169 treatment had less effect on growth and viability during lactation but greater effect on the male reproductive system.

It is evident that the profile of effects seen in PCB 169-treated male and female offspring bears little resemblance to that seen in animals exposed to known antiandrogens. The fact that PCB 169 treatment fails to reduce AGD and induce areolas, retained nipples, and male hypospadias, hallmarks of antiandrogenic action, at a dosage level that produces significant reproductive toxicity suggests that the Ah receptor agonists should not be considered 'anti-androgens', unless it can be shown that the mechanism of action involves alterations in androgen or AR levels or AR–DNA binding. The fact that a compound alters an androgen-dependent tissue does not necessarily indicate that it is antiandrogenic, because many other growth factors (i.e., epidermal growth factor) and hormones (i.e., prolactin, thyroid, and growth hormones) also are required for maximal development of these tissues. In addition, PCB 169 and TCDD induce many effects that are clearly unrelated to androgen action (eye opening, and female effects like cleft phallus and vaginal threads).

In the preceding experiment, we found that males exposed to P during perinatal life had increased incidences of prostatitis and vesiculitis. Here, we report that *in utero* exposure to an Ah receptor agonist also can increase the incidence of prostatitis in the dorsolateral lobe of the prostate in 600-day-old male rats from 2/15 in controls to 7/10 in the treated males ($p < 0.05$) (Table 4). Also, the epididymis of the treated males displayed a nonsignificant increase in the incidence of chronic focal inflammation [6/10 *versus* 4/15 in controls ($p < 0.15$)], and males displayed diffuse epithelial hypertrophy of the ventral prostate (1/9 *versus* 0/15 in control males).

Table 4. The effects of administration of PCB 169 (a single dose of 1.8 mg kg^{-1} on gestational day 8), an Ah receptor agonist, on the reproductive development of pubertal (one male per litter necropsied) and adult male LE hooded rat offspring (all surviving males necropsied)

	Control males (65-day old)	PCB 169 males (65-day old)	Control males (adult)	PCB 169 males (adult)
Ventral prostates (mg)	292 ± 15	118 ± 35; $p < 0.0007$[a]	576 ± 44	357 ± 35; $p < 0.005$[a]
Seminal vesicles (mg)	1163 ± 66	622 ± 13; $p < 0.005$[a]	1647 ± 121	1283 ± 188; $p < 0.12$
Testes (g)	3.38 ± 0.07	2.53 ± 0.38; $p < 0.055$[a]	3.92 ± 0.09	3.37 ± 0.27; $p < 0.07$
Epididymides (mg)	699 ± 25	506 ± 71; $p < 0.03$[a]	1390 ± 46	1207 ± 93; $p < 0.09$
Cauda Epididymides (mg)	135 ± 7.9	89 ± 13.2; $p < 0.015$[a]	314 ± 13	246 ± 42; $p < 0.13$
Epididymal sperm count × 10^6	71.5 ± 4.1	31.0 ± 8.9; $p < 0.002$[a]	277 ± 12	193 ± 30; $p < 0.03$
Testis spermatid count × 10^6	221 ± 5.9	151 ± 30; $p < 0.05$[a]	259 ± 8	202 ± 28; $p < 0.065$
Caudal sperm count × 10^6	no data	no data	151 ± 15	89 ± 27; $p < 0.06$[a]
Fertility	no data	no data	72%	46%
Acute prostatitis: dorsolateral prostate	no data	no data	13.3%	70%; $p < 0.05$[a]
Ejaculated sperm count × 10^6	no data	no data	135 ± 20	23 ± 16; $p < 0.005$[a]
Percentage of hypospadias	0	0	0	0
Number of litters (Males)	7(7)	8(8)	6(19)	5(12)
Percentage of those with permanent nipples	0	0	0	0

[a]Values differ significantly from the control by litter means analysis.

At 65 days of age body, liver, brain, kidney, pituitary, and adrenal weights did not differ between the control and treated groups (data not shown). The adult male data are from animals necropsied at 260 and 600 days of age. The male necropsy at 600 days of age included three males from two PCB 169 litters, weighing 321, 433, and 479 g and displaying reductions of greater than 50% of testis, ventral prostate, and seminal vesicle weights. However, no males exhibited any malformations.

Table 5. The effects of administration of ketoconazole, a chemical that inhibits steroid hormone synthesis, from day 14 of pregnancy to day 3 of lactation on pregnancy and reproductive development of male LE hooded rat offspring*

Effect	Maternal dose (mg kg^{-1} day^{-1})			
	0	12.5	25	50
Number of dams treated	8	8	8	7
Number pregnant	8	7	7	6
Number of dams delivering on time	8	6	2[a]	0[a]
Number of maternal deaths at term	0	0	1	2
Number of pregnant dams with viable litters to day 4	7	6	3[a]	1[a]
Litter size at day 2	13.6 ± 1.1 (8)	10.1 ± 1.7 (7)	7.1 ± 2.2 (7)[a]	8.0 (1)
Male anogenital distance (mm) on day 2	3.14 ± 0.08 (7)	3.44 ± 0.13 (6)	3.13 ± 0.16 (5)	3.49 (1)
Male offspring necropsy data				
Sample size litters (males)	8 (32)	6 (29)	3 (11)	1 (2)
Body weight (g)	572	589	557	552
Liver weight (g)	18.7	18.7	17.6	18.6
Kidney weight (g)	3.47	3.62	3.20	3.32
Adrenal weight (mg)	43.4	49.6	41.8	37.2
Testes weight (g)	3.78	3.91	3.74	3.26 ($p < 0.07$)
Ventral prostate weight (mg)	448	479	407	558
Seminal vesicle weight (mg)	1.80	1.81	1.74	1.40 ($p < 0.03$)
Epididymal weight (mg)	1329	1344	1343	1134 ($p < 0.09$)
Incidence of male reproductive tract malformations	0	0	0	0
Number of retained nipples	0	0	0	0

[a]Differs significantly from control values.
*Probabilities listed for male offspring at 50 mg kg^{-1} day^{-1} are *t*-statistic values; in no case was the overall ANOVA significant for necropsy data.

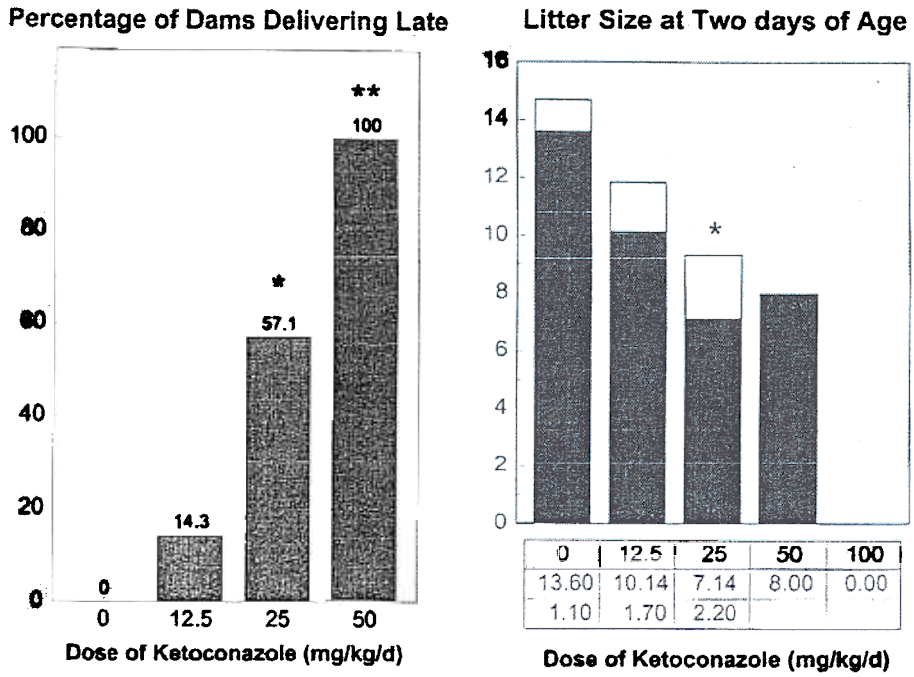
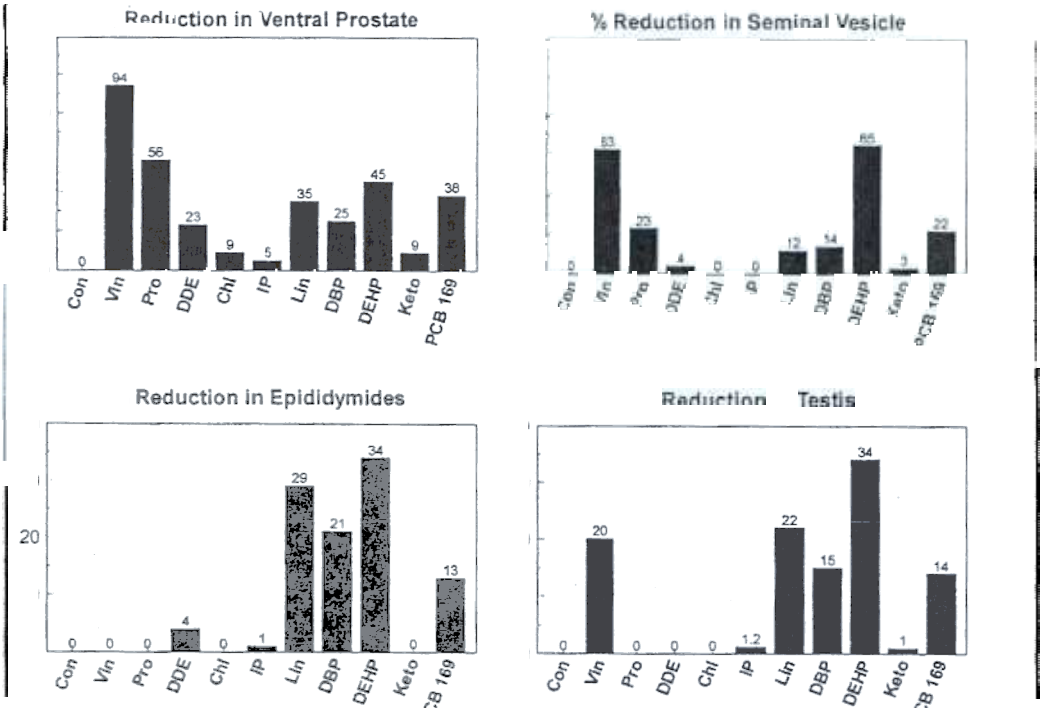


Figure 7. Ketoconazole was administered orally in oil at 12.5, 25, or 50 mg kg^{-1} day^{-1} from gestational day 14 to postnatal day 3. The 100 mg ketoconazole kg^{-1} day^{-1} group was only treated from days 14–18 of gestation. Ketoconazole treatment at 25 and 50 mg kg^{-1} day^{-1} delayed or prevented parturition, resulting in some maternal and extensive neonatal mortality, presumably from an inhibition of ovarian hormone synthesis. In an earlier study, treatment with ketoconazole at 100 mg kg^{-1} day^{-1} caused all of the females to abort during midpregnancy within a few days of the initiation of treatment. Litter sizes at birth were reduced by ketoconazole treatment at 25, 50, and 100 mg kg^{-1} day^{-1}. Male offspring from the 12.5 and 25 mg kg^{-1} day^{-1} groups were not demasculinized or feminized.

Table 6. A comparison of the effects of exposure to potentially antiandrogenic chemicals on the offspring[a]

	Vinclozolin (100–200 mg kg^{-1} day^{-1} on GD14–PND4)	Procymidone (100–200 mg kg^{-1} day^{-1} on GD14–PND3)	*p,p'*-DDE (100 mg kg^{-1} day^{-1} on GD14–GD18)	Linuron (100 mg kg^{-1} day^{-1} on GD14–GD18)	DBP (500 mg kg^{-1} day^{-1} on GD14–PND3 GD16–19)	DEHP (750 mg kg^{-1} day^{-1} on GD14–PND3)	Ketoconazole (12.5, 25, 50, 100 mg kg^{-1} day^{-1} on GD14–PND3)	TCDD (1 μg kg^{-1} d^{-1} on GD 8 or 15)	PCB 169 (1.8 mg kg^{-1} on GD 8)
Rat strain	LE	LE	LE and SD	SD	SD and LE	SD	LE	LE and SD	LE
Maternal death or reduced weight gain				*reduced weight gain*			*death at 50 and 100*		
Reduced anogenital distance at birth	*yes*	*yes*		*yes*	*yes*	*yes*	no	no	no
Areolas in infant male rats	*yes*	*yes*	*yes*	*yes*	*yes*	*yes*	no	no	no
Male hypospadias	*yes*	*yes*	*yes*	*yes*	*yes*	*yes*	no	no	no
Female hypospadias	no	no	no	no	no	no	no	*yes*	*yes*
Male: permanent nipples	*yes*	*yes*	*yes*	*yes*	*yes*	*yes*	no	no	no
Reduced prostate weight	*yes*	*yes*	*yes*	*yes*	*yes*	*yes*	no	*yes*	*yes*
Prostate agenesis	*yes*	*yes*	no	no	no	*yes*	no	no	no
Reduced seminal vesicle weight	*yes*	*yes*	slight	*yes*	*yes*	*yes*	no	*yes*	*yes*
Seminal vesicle agenesis	no	no	no	no	no	*yes*	no	no	no
Reduced testis weight	at 200	no	no	*yes*	*yes*	*yes*	no	*yes*	*yes*
Testis agenesis/atrophy	*atrophy*	no	no	*yes*	*yes*	*yes*	no	no	no
Whole epididymal weight reduced	*yes*	no	no	*yes*	*yes*	*yes*	no	*yes*	*yes*
Epididymal agenesis	not at 200	no	no	*yes*	*yes*	*yes*	no	no	

[a]While this table emphasizes effects seen in male rat offspring, some of these chemicals also alter reproductive function in the female offspring as well, while for other chemicals, the female offspring have not yet been as thoroughly studied. The data for *p,p'*-DDE, linuron, ketoconazole, DBP, DEHP, procymidone, and PCB congener 169 are presented in this manuscript, while the effects of TCDD and vinclozolin are from published studies (Gray et al., 1994, 1995a, 1997a,b; Gray and Ostby, 1995).


Reduction in Ventral Prostate
0
94
56
23
9
5
35
25
45
9
38
Con
Vin
Pro
DDE
Chl
IP
Lin
DBP
DEHP
Keto
PCB 169
% Reduction in Seminal Vesicle
Reduction in Epididymides
20
0
0
0
4
0
1
29
21
34
0
13
Reduction Testis
0
20
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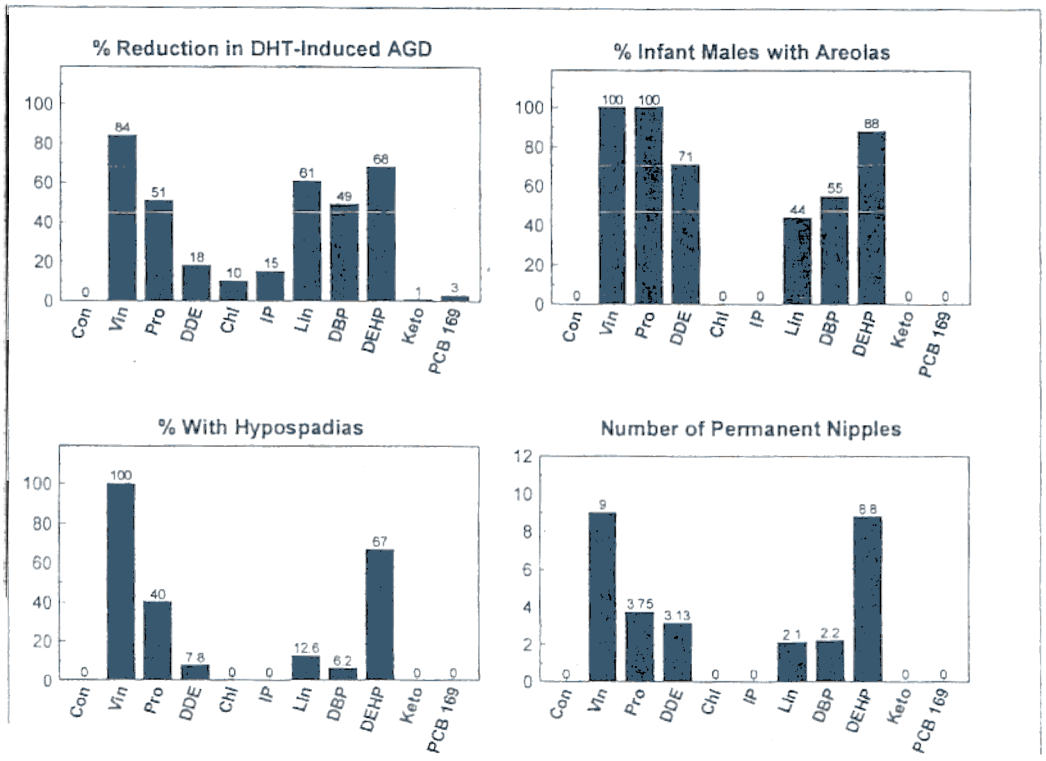
1.2
22
15
34
1
14


% Reduction in DHT-Induced AGD
100
80
60
40
20
0
0
84
51
18
10
15
61
49
68
1
3
% Infant Males with Areolas
100
100
71
0
0
44
55
88
0
0
% With Hypospadias
100
40
7.8
0
0
12.6
6.2
67
0
0
Number of Permanent Nipples
12
10
8
6
4
2
0
9
3.75
3.13
0
0
2.1
2.2
8.8
0
0

Ketoconazole results

Administration of ketoconazole during pregnancy in the rat at 100 mg kg^{-1} day^{-1} leads to reduced maternal weight gain and whole litter loss within a few days of the initiation of treatment on day 14 of pregnancy. These effects are consistent with the ability of this fungicide to inhibit progesterone synthesis. In the current study, ketoconazole treatment delayed the onset of parturition by as much as 3 days and reduced the numbers of live pups at all dosage levels, being statistically significant at 25 and 50 but not 12.5 mg kg^{-1} day^{-1} (Table 5; Figure 7). This suggests that ketoconazole inhibits the synthesis of estradiol near-term, possibly by inhibiting aromatase activity (another cytochrome P-450). The fungicide, fenarimol, is known to inhibit aromatase activity *in vitro* and it also delays delivery and induces maternal death (Gray and Ostby, 1998). Our postnatal data indicate that T synthesis in the fetal male is not inhibited at these dosage levels. Male pups did not display an indication of being demasculinized or feminized by ketoconazole treatment (Table 5). As the specificity for different cytochrome P-450 enzymes in the steroid pathway varies from compound to compound, it is impossible to generalize about the sensitivity of pregnancy, parturition, and sexual differentiation to disruption by these fungicides.

Ethane dimethane sulphonate (EDS) results

The current experiment was designed to determine if oral perinatal maternal treatment with EDS would demasculinize male rat offspring. Treatment was terminated at day 21 of gestation because the EDS-treated dams' body weights were reduced compared to their body weights on the 14th day of pregnancy, while control dams had gained over 60 g each over the same period of gestation. One EDS-treated dam delivered seven dead pups and then died on day 3 of lactation. Another dam was unable to deliver her entire litter and was euthanized on day 3 of lactation. Three litters contained live pups on day 1 when AGD was measured, however, none of these pups survived to 4 days of age. The numbers of pups born live did not differ significantly between the groups, being 11.4 ($n = 5$) for controls *versus* 9.7 for the EDS group ($n = 3$). Body weight was significantly reduced by 45%, from 6.21 g in controls to 3.4 g ($p < 0.01$), by EDS treatment. In contrast, AGD development was spared as compared to general somatic growth, being reduced by only 13.1% from 3.09 mm (control males) to 2.68 mm (EDS males). Apparently, the physiological alterations associated with EDS-induced maternal toxicity and reduced pup growth and viability did not include an equivalent inhibition of testicular T production or the conversion of T to DHT in the anogenital area. It is useful to note that calculation of the AGD Index (AGD/body weight) as advocated by some in the field, would incorrectly imply that the EDS males were masculinized by EDS treatment. AGD Index was increased by 58% from 0.497 in controls to 0.788 in the EDS-treated group. These results clearly demonstrate that general maternal and neonatal toxicity do not result in demasculinization of AGD.

Discussion

An analysis of the data in these studies indicates that the chemicals included in our investigation can be clustered into three or four distinct groups based on the resulting profiles of reproductive effects (Table 6). Vinclozolin, P, and DDE, known AR ligands, produce similar profiles of toxicity, however, *p,p'*-DDE is less potent in this regard. While V and P produce a typical 'flutamide-like' profile (ventral prostate agenesis and undescended testes), *p,p'*-DDE produces a profile that resembles that obtained with either low dosage levels of V, P, or flutamide or an inhibitor of DHT synthesis. DBP and DEHP, which do not appear to be AR ligands, produce profiles distinct from the AR antagonists. Our results are consistent with those of Mylchreest et al. (1998); male offspring display a higher incidence of epididymal and testicular lesions than generally seen with flutamide, P, or V even at high dosage levels (Figure 8). L, an AR ligand, produced a profile that we found to be most surprising. L treatment induced a level of external effects consistent with its low affinity for AR (reduced AGD and nipples and a low incidence of hypospadias); however, L treatment induced an unanticipated degree of malformed epididymides and testicular

Figure 8 (from previous page). This figure displays the effects of perinatal administration of vinclozolin (VIN), procymidone (PRO), *p,p'*-DDE (DDE), chlozolinate (CHL), iprodione (IP), linuron (LIN), dibutyl phthalate (DBP), diethylhexyl phthalate (DEHP), ketoconazole (KETO) and PCB congener 169 (PCB 169) on several dihydrotestosterone (DHT)- and testosterone-dependent tissues. The data are displayed as percentage of concurrent control. For anogenital distance (AGD), it is assumed that DHT is responsible for half of AGD length, as female rats have an AGD half the size of male rat pups. In this regard, a reduction in DHT-induced AGD of 84% indicates that measured AGD was reduced by 42% of the concurrent control, a value that was doubled in order to indicate the degree to which DHT was inhibited. CHL, IP, and KETO did not alter male rat sexual differentiation at the dosage levels used in the current study. LIN, DBP, and DEHP induced greater effects on the epididymis and testis than did VIN, PRO, and DDE (the typical profile for androgen receptor antagonists). Of all of the developmental reproductive toxicants, only PCB 169 did not reduce AGD, nor did it induce areolas in infant male ra **ypospadias, or permanent nipples.**

atrophy. In fact, the profile of effects induced by L was quite similar to that seen with DBP. Both L and DBP display antiandrogenic activity but only L appears to be an AR antagonist. These results suggest that L may display several mechanisms of endocrine toxicity, one of which involves AR binding.

Several of the chemicals studied here produced some form of maternal or neonatal toxicity, including EDS, ketoconazole, *p,p'*-DDE, and L. The EDS data indicate that extreme reductions in health and size of the neonates are accompanied by a lesser effect on AGD. The ketoconazole results demonstrate that even when a compound produces maternal toxicity and delays parturition, surviving male pups are not necessarily demasculinized or feminized. Ketoconazole-treated males pups had normal AGD, they lacked areolas or retained nipples, and did not display malformations of androgen-dependent tissues.

Comparison of the effects of developmental reproductive toxicants using a pseudohermaphroditism index (PHI)

In an earlier study (Gray et al., this issue), we created a descriptive variable termed the 'pseudohermaphroditism index' in order to visualize the dose-related effects of V

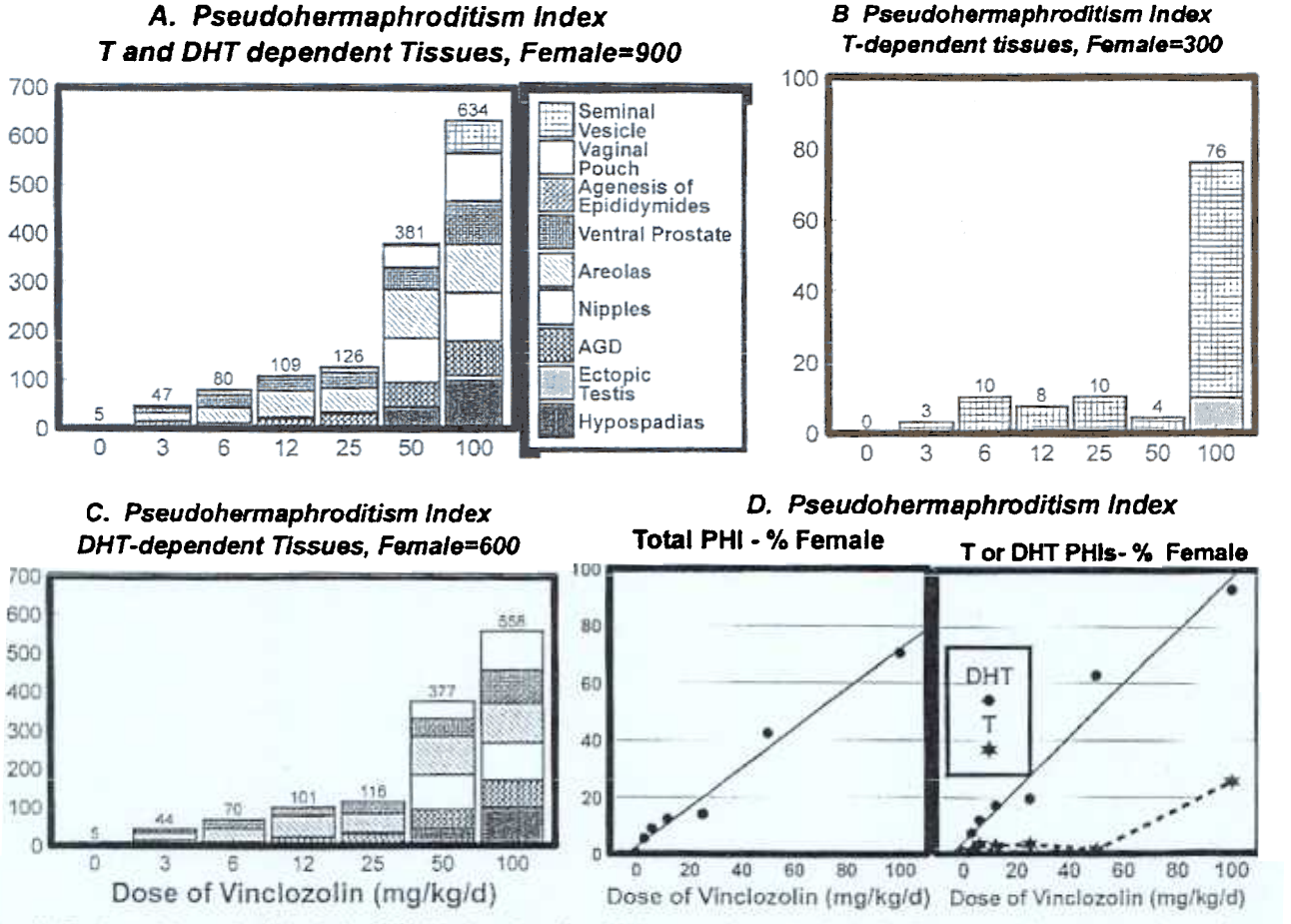


Figure 9. The 'pseudohermaphroditism index (PHI)' is altered in a female-like manner by perinatal vinclozolin treatment (3–100 mg kg^{-1} day^{-1} from day 14 of pregnancy to day three of lactation) (Gray et al., this issue). It is our intent that this index be used to compare the overall profiles or 'fingerprints' of the reproductive alterations produced by different chemicals; the implication being that a different profile likely results from a different mechanism of action. In the upper left panel, the PHI is calculated based on the percent reduction of nine androgen-dependent variables. In the panel on the upper right, the PHI is calculated for three T-dependent tissues, while the panel on the lower left displays the PHI for six dihydrotestosterone (DHT)-dependent tissues. The panel on the lower right indicates that total PHI and DHT-PHI are linear across the dose range used in our study on vinclozolin, reflecting the fact that many of the DHT-dependent variables fail to display a discernible threshold. In contrast, for vinclozolin, the T-dependent responses across dose display an apparent threshold. The results from our vinclozolin dose–response study are used in the next figure to compare the effects of the chemicals in the current study to a known androgen receptor antagonist.

across multiple reproductive tissues (Figure 9). This index combines the degree of feminization/demasculinization attained by V treatment on several androgen-dependent tissues (AGD, areolas, retained nipples, ventral prostate weight, seminal vesicle weight, percent with hypospadias, percent with testicular ectopia, percent with vaginal pouch, and percent with epididymal agenesis) at each dosage level. A PHI score of zero indicates that the group has a male phenotype with respect to androgen-dependent tissues, while a PHI score of 900 indicates that the males were completely feminized and demasculinized by treatment (i.e., the AGD was of female length, all males displayed areolas, twelve retained nipples, hypospadias, a vaginal pouch, paired undescended testes, and complete agenesis of the ventral prostate, seminal vesicle, and epididymis). In other words, a group of males with a PHI score of 900 would resemble phenotypic females in regards to androgen-dependent tissues. As seen in Figure 9, male pups exposed to 100 mg V kg^{-1} day^{-1} were about 70% female-like in their phenotype. DHT-dependent tissues were much more affected than were T-dependent tissues, being inhibited by 90% *versus* 25%, respectively. In addition, DHT-dependent tissues appeared to be altered in a linear fashion, while T-dependent tissues displayed a threshold, being only marginally affected at 50 mg V kg^{-1} day^{-1} and below. This profile is typical for an AR antagonist.

Here, we utilized this index to describe how the profiles of reproductive effects in males exposed to *p,p'*-DDE, P, L, DBP, DEHP, or PCB 169 compared to V (Figure 10). For each compound, two PHI scores were calculated, one for DHT (AGD, areolas, retained nipples, ventral prostate size, hypospadias, and vaginal pouches) and the other for T-dependent tissues (seminal vesicle, epididymal, and testicular size or percent abnormal). In calculation of the DHT-PHI score for PCB 169, *p,p'*-DDE, and P, which did not induce malformations in the testes or epididymides, we used the reduction in organ weight relative to the control value to differentiate between compounds that permanently reduced the size of the organs without causing malformations from those that did not reduce organ size.

Figure 10 was constructed to provide a visual summary of the results of the data collected on multiple androgen-dependent tissues and to compare these results to the previously reported dose–response effects of V on the PHI (Gray et al., this issue). Procymidone and *p,p'*-DDE, which are known AR antagonists, produced profiles that resemble that produced by V although they were obviously less potent (Ostby et al., this issue). In contrast, the phthalates DBP and DEHP produced higher incidences of testicular and epididymal abnormalities than was anticipated based on the DHT-dependent external effects which make up 5th/6th of the T-PHI score. The weak AR agonist L probably provided one of the greatest surprises in the current study. Based on a external examination of the animals (AGD, areolas, nipples, hypospadias, and vaginal pouch), we did not expect to see any endocrine-disrupting effects on the epididymides or testes. The fact that these tissues were affected to a high degree suggests that L either alters sexual differentiation by another mechanism

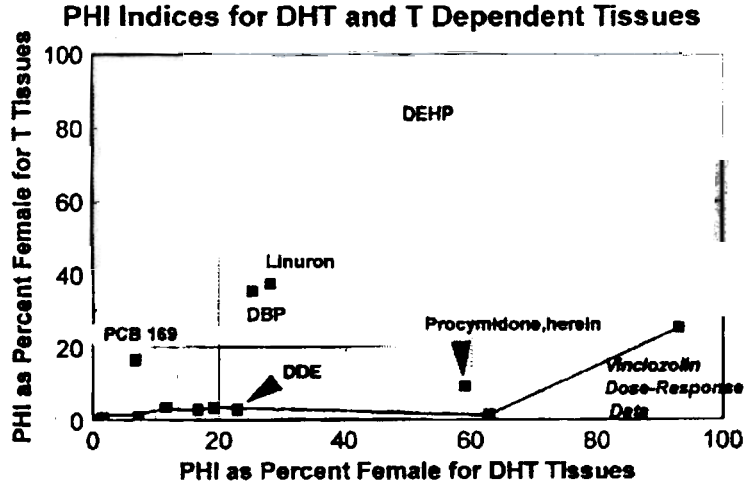


Figure 10. This figure presents a comparison of the effects of different potentially antiandrogenic chemicals on testosterone (T)- and dihydrotestosterone (DHT)-dependent fetal tissues. A pseudohermaphroditism index (PHI) of 100% indicates that the male was completely demasculinized and feminized in regards to the tissues examined. Many of the compounds used in this investigation produce responses that differ from the standard vinclozolin model, indicating that other mechanisms of action may be involved in altering fetal male reproductive tissues.

of action in addition to being an AR antagonist or the epididymides concentrate the compound or its active metabolites. PCB 169 treatment had no effect on the external androgen (DHT)-dependent tissues (AGD, areolas, retained nipples, or external malformations), but it did affect ventral prostate size (DHT-dependent) and epididymal (T-dependent) and testis size. The fact that the development of these tissues are, androgen-dependent, does not necessarily indicate that this is the mechanism of toxicity for PCB 169, because abnormal development of these tissues also can result from other molecular mechanisms.

Summary

We show here that pesticides and toxic substances can alter sexual differentiation in an antiandrogenic manner via several distinct mechanisms. This must be considered in the selection of assays for screening for anti-androgens as proposed by the Endocrine Disruptor Screening And Testing Advisory Committee (EDSTAC). Assays for the detection of antiandrogenicity must be sufficiently comprehensive to enable them to detect the antiandrogenic activity of chemicals that act by all mechanisms of action, not just those that act by binding the AR. A screening battery for antiandrogenicity should be able to detect DBP, DEHP, and L, as well as P and V. As currently proposed, the EDSTAC Screening Battery does not include *in utero* exposures. It relies upon (1) *in vitro* AR binding or transcriptional activation, (2) an *in vitro* assay of steroidogenesis using minced testis culture, and (3) a short-term *in vivo* assay that is designed to detect anti-androgens (or androgens) by measuring androgen-dependent organ weights in castrate-testosterone-treated males. Another *in vivo* assay, termed the 'pubertal male assay,' was proposed as an alternative *in vivo* assay. The pubertal male assay examines the effects of potential endocrine disruptors on pubertal development in the male rat (Monosson et al., this issue).

If it is determined that these assays fail to detect the endocrine activity of chemicals that alter reproductive development, then renewed consideration should be given to utilization of a short-term *in utero* assay. Our results indicate that chemicals that interfere with steroid hormone synthesis, like ketoconazole or fenarimol, could be detected within a few days if treatment was initiated at mid-pregnancy (about ten litters per group) and continued through lactation. Antiandrogenic chemicals could be detected by their effects on AGD at 2 days of age and induction of areolas in infants (when a positive response was detected in these two tissues the offspring could be retained for further observation). In addition, alterations of thyroid hormone homeostasis could be detected by examining serum thyroxine, triiodothyronine, and thyroid stimulating hormone and thyroid histology in 2- to 3-week-old offspring and their dams. The dosing phase of this assay would be about 30 days if pups were maintained until weaning. Short-term *in utero* assays like that described here were considered by EDSTAC (see the Alternative Reproductive Mammalian Test in the EDSTAC Final Report, Chapter Five, Appendix Q, pp. 4–6). However, at the time of their deliberations, insufficient data were available to evaluate their utility. In this regard, our study presents information that indicates that such an assay is feasible and its implementation, after standardization and validation, would eliminate concerns that a Screening Battery that does not include *in utero* exposure will allow chemicals that alter reproductive development by endocrine mechanisms to elude detection.

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Androgen Receptor Antagonist *versus* Agonist Activities of the Fungicide Vinclozolin Relative to Hydroxyflutamide*

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The mechanism of antiandrogenic activity of vinclozolin (3-(3,5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-2,4-dione), a dicarboximide fungicide under investigation for its potential adverse effects on human male reproduction, was investigated using recombinant human androgen receptor (AR). The two primary metabolites of vinclozolin in plants and mammals are M1 (2-[[3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid) and M2 (3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide). Both metabolites, in a dose-dependent manner, target AR to the nucleus and inhibit androgen-induced transactivation mediated by the mouse mammary tumor virus promoter. M2 is a 50-fold more potent inhibitor than M1 and only 2-fold less than hydroxyflutamide. In the presence of dihydrotestosterone (50 nM), M2 (0.2–10 μM) inhibits androgen-induced AR binding to androgen response element DNA. In the absence of dihydrotestosterone, concentrations of 10 μM M2 or hydroxyflutamide promote AR binding to androgen response element DNA and activation of transcription. Agonist activities of M2 and hydroxyflutamide occur at 10-fold lower concentrations with the mutant AR (Thr^{877} to Ala) endogenous to LNCaP human prostate cancer cells. The results indicate that androgen antagonists can act as agonists, depending on ligand binding affinity, concentration, and the presence of competing natural ligands.

The human androgen receptor (AR)[1] is a member of the steroid hormone receptor family of ligand-activated transcriptional regulatory proteins required for normal male sex development. Androgens through their receptor stimulate masculinization of the fetus and induce male imprinting of the developing brain. Molecular defects in the AR gene cause the syndrome of androgen insensitivity, which results from failure of AR androgen binding, nuclear import, DNA binding, and/or transcriptional activation (1). Certain antiandrogens, such as hydroxyflutamide, bind AR with moderate affinity, promote nuclear import (2), but inhibit androgen-mediated transcriptional activity by failing to promote DNA binding, whereas others, such as cyproterone acetate, promote DNA binding at moderate concentrations and induce partial agonist activity (3).

Vinclozolin is a dicarboximide fungicide registered in the United States and Europe for use on fruits, vegetables, ornamental plants, and turf grasses. Administration of vinclozolin to adult male rats causes Leydig cell hyperplasia and atrophy of the prostate and seminal vesicles (4), whereas administration to pregnant rats causes incomplete development of the male reproductive tract (*i.e.* cleft phallus and hypospadias) in male pups (5, 6), indicating antiandrogenic activity. Two major ring-opened metabolites of vinclozolin (*i.e.* the butenoic acid M1 and the enanilide M2, Fig. 1) predominate in plants and soil (7–9) as well as in rodent fluid and tissue extracts following *in vivo* exposure (10, 11). It was shown previously that vinclozolin, M1, and M2 have little effect on the androgen-metabolizing enzyme 5α-reductase. In addition, vinclozolin was a poor inhibitor of androgen binding to rat AR in cell-free extracts, whereas M1 and M2 were effective competitors (10), suggesting that the antiandrogenic effects of vinclozolin are mediated by M1 and/or M2. It has not been established, however, the degree to which environmental levels of vinclozolin, M1, or M2 induce adverse developmental effects.

In this report, the mechanism of transcriptional inhibition by vinclozolin and its metabolites is shown to be inhibition of androgen-induced DNA binding and subsequent transactivation. A surprising and important result of the study is that at high concentrations in the absence of DHT, vinclozolin metabolite M2 and the classical androgen antagonist metabolite, hydroxyflutamide, are agonists, since they increase AR DNA binding and transcriptional activity. The results reveal that the androgen antagonist activities of these nonsteroidal aromatic compounds are concentration-dependent and may be influenced by the binding of native androgens to the AR dimer.

EXPERIMENTAL PROCEDURES

Materials—Monkey kidney COS-1 and CV1 cells were from the American Type Culture Collection; Dulbecco's modified essential medium with high glucose with or without phenol red, Grace medium supplemented with yeastolate and lactalbumin hydrolysate, and Ex-cell 400 or 401 with L-glutamine were from JRH Biosciences; fetal calf serum for mammalian and insect cell cultures were from Life Technologies, Inc.; *Spodoptera frugiperda* Sf9 cells were from Invitrogen Corp., San Diego CA; antibiotics and gentamicin were from Life Technologies, Inc.; D-luciferin was from Analytical Luminescence; CV1 cell lysis buffer was from Ligand Pharmaceuticals; [^{3}H]methyltrienolone ([17α-*methyl*-^{3}H]R1881, 85.5 Ci/mmol) was from DuPont NEN; Texas red-conjugated goat anti-rabbit IgG was from Molecular Probes, Inc., Eugene OR;

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[1] The abbreviations and trivial names used are: AR, androgen receptor; vinclozolin, (3-(3, 5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-2,4-dione; M1, (2-[[3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid; M2, 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide; R1881, methyltrienolone; PBS, phosphate-buffered saline; DHT, dihydrotestosterone; LNCaP, lymph node-derived human prostate carcinoma cell line; hydroxyflutamide, α,α,α-trifluoro-2-methyl-4′-nitro-*m*-lactotoluidide (SCH16423).

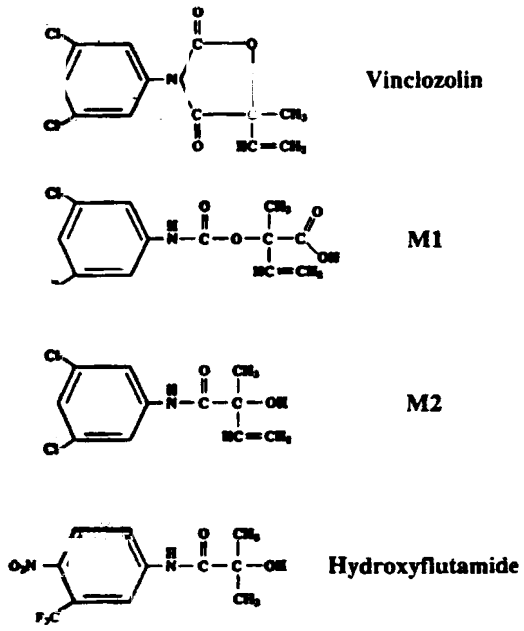

FIG. 1. **Structural formulas of vinclozolin, metabolites M1 and M2, and hydroxyflutamide.**

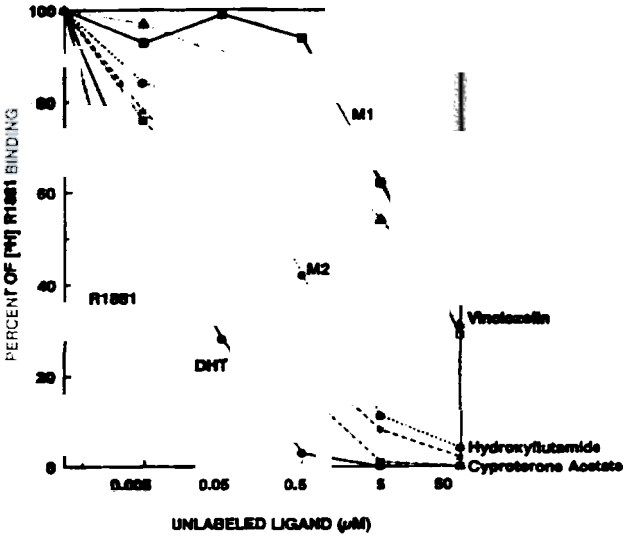

FIG. 2. **Competitive inhibition of [³H]R1881 binding to AR by unlabeled vinclozolin, its metabolites M1 and M2, and hydroxyflutamide and cyproterone acetate.** Binding inhibition was determined in COS cells transiently transfected with the human AR expression vector, pCMVhAR, as described under "Experimental Procedures." Results expressed as percent binding relative to [³H]R1881 alone are shown for unlabeled R1881 (▲), dihydrotestosterone (DHT, ●), cyproterone acetate (■), hydroxyflutamide (*), M2 (–●–), vinclozolin (△), and M1 (□) and are representative of three independent experiments.

vinclozolin was from Crescent Chemical Co., Hauppauge, NY; vinclozolin metabolite M2 was from BASF AG; vinclozolin metabolite M1 was synthesized using alkaline hydrolysis of vinclozolin and purified as described previously (10); hydroxyflutamide was provided by R. O. Neri, Schering Corp., Bloomfield, NJ; unlabeled steroids were from Sigma, and buffers and chemicals were from Fisher, EM Science, and Sigma.

Vinclozolin Metabolism—Serum and cell extract concentrations of vinclozolin, M1, and M2 were determined by high-performance liquid chromatography/diode array detection as described previously (7–10) using linuron (*N'*-(3,4-dichlorophenyl)-*N*-methoxy-*N*-methylurea) as internal standard to correct for procedural losses. In animal studies, adult male rats were dosed orally with 30 or 100 mg of vinclozolin in corn oil/kg of body weight/day or with vehicle alone for 30 days, after which time the animals were killed. Care of the animals was in accordance with institutional guidelines.

Competitive Steroid Binding Assays—Whole cell binding assays were performed as described previously (12). COS-1 cells (10^6/well of 12-well plates) were transfected with 1 μg of pCMVhAR DNA/well using diethylaminoethyl dextran. Twenty-four h prior to the binding reaction, cells were placed in serum-free, phenol red-free medium and incubated for 2 h at 37 °C with 5 nM [³H]R1881 in the presence and absence of increasing concentrations of unlabeled compounds. Nonspecific binding of [³H]R1881 was assessed by adding a 100-fold molar excess of unlabeled R1881. Cells were washed twice in phosphate-buffered saline (PBS), harvested in 200 μl of 2% SDS, 10% glycerol, and 10 mM Tris, pH 6.8, and radioactivity determined by scintillation counting.

Immunocytochemistry—COS cells (10^5 cells/well of two-chamber slide) were transfected with 1 μg/well pCMVhAR using diethylaminoethyl dextran as described previously (13). Hormones were added 24 and 1 h prior to washing with PBS. Cells were air-dried, fixed in 95% ethanol at −20 °C for 10 min, washed in PBS, treated in 5% bovine serum albumin in PBS, pH 7.4, for 30 min, and incubated with AR52 IgG (2 μg/ml) overnight at 4 °C. Cells were washed in PBS and incubated with Texas red-conjugated goat anti-rabbit IgG (1:500) for 60 min at room temperature. The stained cells were examined using a Nikon Optiplot 2 microscope with an EP1 fluorescence attachment.

Androgen-dependent Transcription Assays—Transcriptional activity was assessed by transient cotransfection of monkey kidney CV1 cells (0.4×10^6/6-cm dish) with 50–100 ng of pCMVhAR expression vector and 5 μg of mouse mammary tumor virus luciferase reporter vector (provided by Ronald M. Evans, Salk Institute, La Jolla, CA) using the calcium phosphate precipitation method (13, 14). Twenty-four and 48 h after transfection, the indicated concentrations of DHT and antiandrogens were added with fresh medium and 5 h after the last addition, cells were harvested in 0.6 ml of lysis buffer. Relative light units of a 0.1-ml aliquot were determined using a Monolight 2010 Analytical Luminescence Laboratory luminometer.

DNA Mobility Shift Assays—Sf9 insect cells expressing recombinant full-length wild-type and mutant LNCaP prostate cancer cell line human AR baculoviruses were incubated for 42 h at 26 °C with the indicated concentrations of ligands; cells were harvested and extracted in a high salt containing buffer as described previously (3). The ^{32}P-labeled 27-base pair oligonucleotide contained the androgen response element of the 0.5 kb first intron region of the rat prostatein C3 subunit gene (15) and was analyzed in the DNA mobility shift assay as described previously (3).

RESULTS

Competitive Binding and Metabolism—The molecular basis for the antiandrogenic effects of vinclozolin was investigated using recombinant human AR transiently expressed in monkey kidney COS cells. In a competitive androgen binding assay using [³H]R1881 (a radiolabeled synthetic androgen), M2 was a slightly weaker competitor than the antiandrogen, hydroxyflutamide, but considerably more effective than M1 or vinclozolin (Fig. 2). Half-maximal inhibition of 5 nM [³H]R1881 binding occurred at approximately 500 nM M2 and 10–20 μM M1 or vinclozolin. Strongest competitive binding was observed with the synthetic androgen R1881 and the natural androgen, DHT. Competitive inhibition by cyproterone acetate was greater than that of M2 or hydroxyflutamide. The similar effectiveness of M2 and hydroxyflutamide in competing for [³H]R1881 binding to AR is supported by structural similarity between these two nonsteroidal aromatic compounds (see Fig. 1).

Metabolism of vinclozolin, M1, and M2 was assessed by high-performance liquid chromatography with on-line uv absorbance diode array detection as described under "Experimental Procedures." During a 24-h 37 °C incubation at 50 μM, vinclozolin was metabolized to 87% M1 and 12% M2 in monkey kidney COS cells and 94% M1 and 6% M2 in monkey kidney CV1 cells, the cell lines used to assess AR ligand binding and transcriptional activity, respectively. M1 and M2 were stable during the incubations, with more than 98% of the original compounds retained during the cell cultures. A similar pattern of vinclozolin metabolism was observed in Sf9 insect cells used

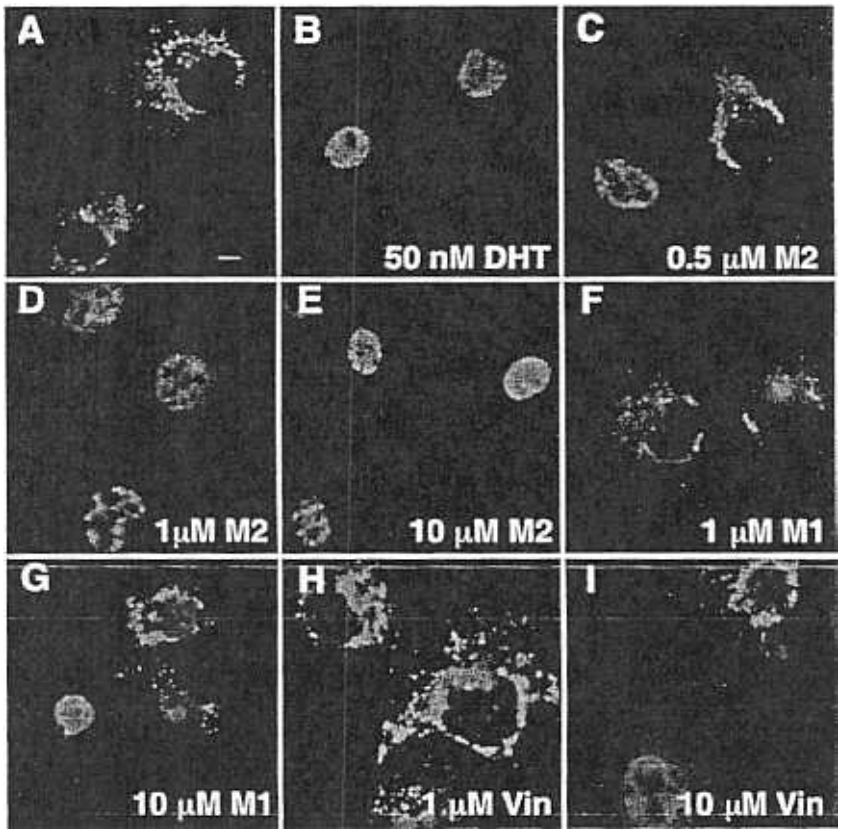

FIG. 3. **Immunocytochemical staining of AR in transfected COS cells in the presence of dihydrotestosterone, vinclozolin, or its metabolites, M1 and M2.** Immunocytochemical staining was performed as described under "Experimental Procedures." Cells expressing AR were untreated (*A*) or exposed for 24 h at 37 °C to 50 nM dihydrotestosterone (*DHT, B*), 0.5 μM M2 (*C*), 1 μM M2 (*D*), 10 μM M2 (*E*), 1 μM M1 (*F*), 10 μM M1 (*G*), 1 μM vinclozolin (*Vin, H*), and 10 μM vinclozolin (*Vin, I*). The regions shown are representative of the overall staining pattern determined in four experiments. Magnification: × 720.

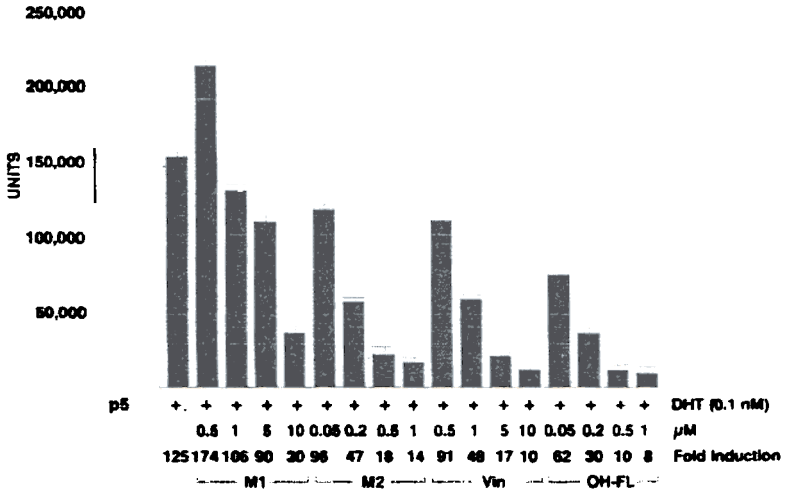

FIG. 4. **Transcriptional inhibitory effects of increasing concentrations of vinclozolin, metabolites M1 and M2, and hydroxyflutamide on DHT-induced transcriptional activity.** Transcriptional activity was determined in transiently transfected CV1 cells as described under "Experimental Procedures" at 0.1 nM DHT and the indicated ligand concentrations. Optical readings are shown with standard error, and -fold induction is indicated at the bottom relative to the activity determined in the absence of DHT. p5 represents results obtained when the parent expression vector pCMV5 lacking AR sequence which was cotransfected with the luciferase reporter vector. The data shown are representative of at least four independent determinations.

for the expression of recombinant baculovirus. Thus, vinclozolin metabolism in primate and insect cells parallels that observed in rats, where M1 and M2 are the predominant metabolites of the fungicide.

Subcellular Distribution—AR transiently expressed in COS cells is perinuclear in the cytoplasm in the absence of androgen and nuclear in the presence of 50 nM DHT as reported previously (13, 14) and shown in Fig. 3, *A* and *B*. Significant AR nuclear staining (40–50%) was observed with exposure to 0.5 μM M2 (Fig. 3*C*), 10 μM M1 (Fig. 3*G*), and 10 μM vinclozolin (Fig. 3*I*), closely paralleling the ability of these ligands to compete for AR androgen binding (see Fig. 2). M1 and vinclozolin at 1 μM resulted in absence of detectable nuclear staining (Fig. 3, *F* and *H*), whereas 1 μM M2 caused nearly complete AR nuclear localization (Fig. 3*D*). At 10 μM, M2 AR nuclear transport was indistinguishable from that observed with 50 nM DHT (Fig. 3, *E* and *B*).

Mechanism of AR Inhibition by Vinclozolin—The effect of vinclozolin and its metabolites on DHT-induced transcriptional activity was investigated by transient cotransfection of monkey kidney CV1 cells with the human AR expression vector and a mouse mammary tumor virus promoter-luciferase reporter vector used previously to measure androgen-induced transcriptional responses to AR (13). Transcriptional activity induced with 0.1 nM DHT was inhibited about 80% by 10 μM M1, 0.2 μM M2, 1 μM vinclozolin, or 0.2 μM hydroxyflutamide (Fig. 4). The

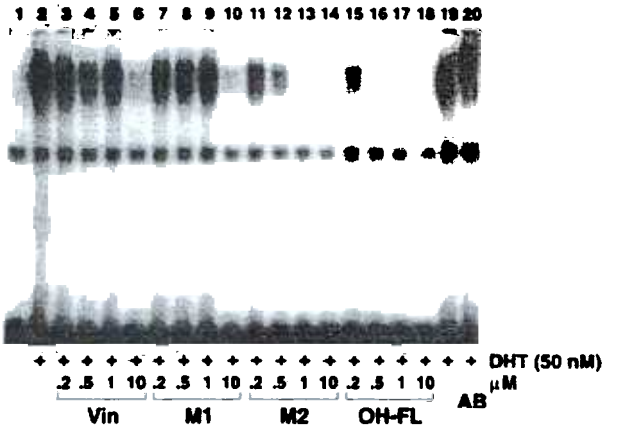

FIG. 5. **Inhibition of DNA binding by baculovirus-expressed recombinant AR.** Sf9 cells expressing wild-type AR were incubated in the absence (*lane 1*) or presence of 50 nM dihydrotestosterone (*DHT*) either alone (*lanes 2, 19,* and *20*) or with increasing concentrations of vinclozolin (*Vin, lanes 3–6*), M1 (*lanes 7–10*), M2 (*lanes 11–14*), or hydroxyflutamide (*OH-FL, lanes 15–18*). DNA mobility shift assays were performed with an androgen response element as described under "Experimental Procedures." The *upper band* represents specific AR binding to ^{32}P-labeled androgen response element DNA and is shifted to a slower migration (*lane 20*) with the addition of AR52 antipeptide antibody (*AB*) described previously (29). The *middle bands* represent nonspecific DNA binding and are detected with extracts from Sf9 cells not exposed to recombinant baculovirus (not shown). At the *bottom* is the *upper portion* of the free labeled oligonucleotide band. The concentration of competing unlabeled ligand ranged from 0.2 to 10 μM as indicated. The data shown are representative of three independent experiments.

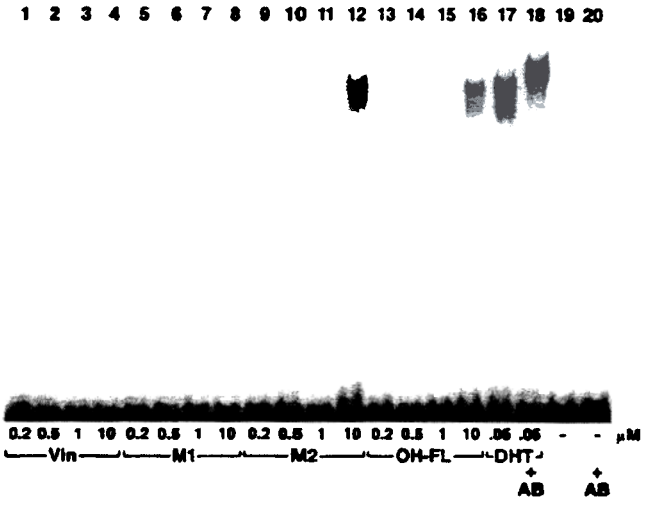

FIG. 6. **Enhancement of AR DNA binding by high concentrations of M2 and hydroxyflutamide in the absence of DHT.** Incubations of Sf9 cells expressing AR were performed in the absence (*lanes 19* and *20*) or presence of 0.05 μM dihydrotestosterone (*DHT, lanes 17* and *18*) or at increasing concentrations of vinclozolin (*Vin, lanes 1–4*), metabolites M1 (*lanes 5–8*), M2 (*lanes 9–12*), or hydroxyflutamide (*OH-FL, lanes 13–16*) between 0.2 and 10 μM. The DNA mobility shift assay was performed as described under "Experimental Procedures." The *upper band* is specific for AR·DNA complex formation, since it is shifted to a slower migration with the addition of antibody AR52 (*AB, lane 18*) but is undetectable in the absence of DHT (*lane 20*). The data shown are representative of four independent experiments.

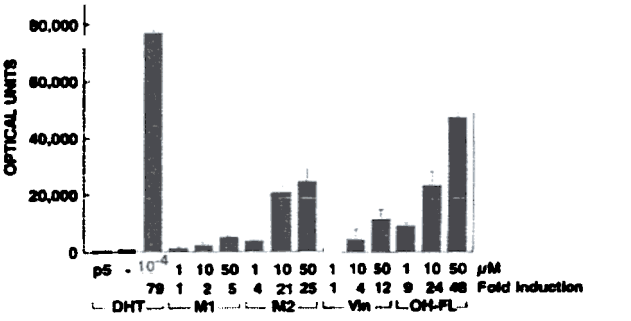

FIG. 7. **Agonist activity of high concentrations of vinclozolin, its metabolites, and hydroxyflutamide with wild-type AR.** Agonist activity was determined by transient cotransfection using the parent expression vector lacking AR coding sequence (p5) or pCMVhAR coding for wild-type AR and the luciferase reporter vector as described under "Experimental Procedures" and in the legend of Fig. 4. Shown are the optical units obtained following incubations with 0.1 nM DHT and increasing concentrations of metabolites M1 and M2, vinclozolin (*Vin*), and hydroxyflutamide (*OH-FL*) in a range from 1 to 50 μM. -Fold induction was determined relative to the activity observed in the absence of added ligand and is shown numerically at the *bottom*.

greater transcriptional inhibition by M2 and hydroxyflutamide compared with vinclozolin and M1 parallel their more effective competitive inhibition of [^{3}H]R1881 binding to AR (see Fig. 2) and their ability to promote AR nuclear transport (see Fig. 3 and Ref. 2). The parent compound vinclozolin and the major metabolite M1 were about 10- and 100-fold less potent, respectively, than M2 in inhibiting AR transcriptional activity (Fig. 4).

The intermediate effectiveness of vinclozolin relative to M1 and M2 in inhibiting DHT-induced transcription in the cotransfection assay (Fig. 4) is consistent with its metabolism to M1 and M2 in CV1 cells (94% M1 and 6% M2). In adult male rats treated orally with 30 and 100 mg of vinclozolin/kg of body weight/day for 30 days, the major serum metabolite was M1; serum levels averaged 107 nM and 1.6 μM vinclozolin, 1.7 μM and 10 μM M1, and 22 nM and 270 nM M2, respectively. The higher concentration of the less potent metabolite M1 suggests that it contributes, with M2, to the antiandrogenic effects of vinclozolin.

The mechanism of transcriptional inhibition was investigated by determining the effect of each ligand on androgen-induced AR binding to androgen response element DNA. It was shown previously that human AR expressed from baculovirus in Sf9 cells requires intracellular exposure to androgen to induce high affinity, sequence-specific DNA binding activity (3) as shown in Fig. 5 (*lanes 1* and *2*). Inhibition of DNA binding induced by 50 nM DHT required 10 μM vinclozolin (Fig. 5, *lane 6*), 10 μM M1 (Fig. 5, *lane 10*), or 0.2–0.5 μM M2, with essentially complete inhibition at 1–10 μM M2 (Fig. 5, *lanes 11–14*). M2 was 2–3-fold less effective than hydroxyflutamide in blocking androgen-induced AR DNA binding (Fig. 5, *lanes 15–18*). The results suggest that the mechanism of antagonism by these ligands is a concentration-dependent inhibition of androgen-induced AR DNA binding, with M2 being the most effective antiandrogen of the vinclozolin metabolites.

Antagonist versus Agonist Activity—When M2 and hydroxyflutamide were added at high concentrations (10 μM) in the absence of androgen to Sf9 cells expressing AR, AR DNA binding was observed (Fig. 6, *lanes 12* and *16*) raising the possibility that these antagonists are agonists at high concentrations in the absence of androgen. (Note the absence of AR DNA binding at these concentrations in Fig. 5 in the presence of DHT.) The luciferase transcription assay was therefore repeated using high concentrations of these compounds in the absence of DHT. As shown in Fig. 7, concentrations of 10 and 50 μM M2 or hydroxyflutamide, and to a lesser extent, vinclozolin, induced AR-mediated transcriptional activation from 20- to 48-fold. In the absence of AR expression, no agonist effects were observed, ruling out inductions through non-AR-mediated pathways. The

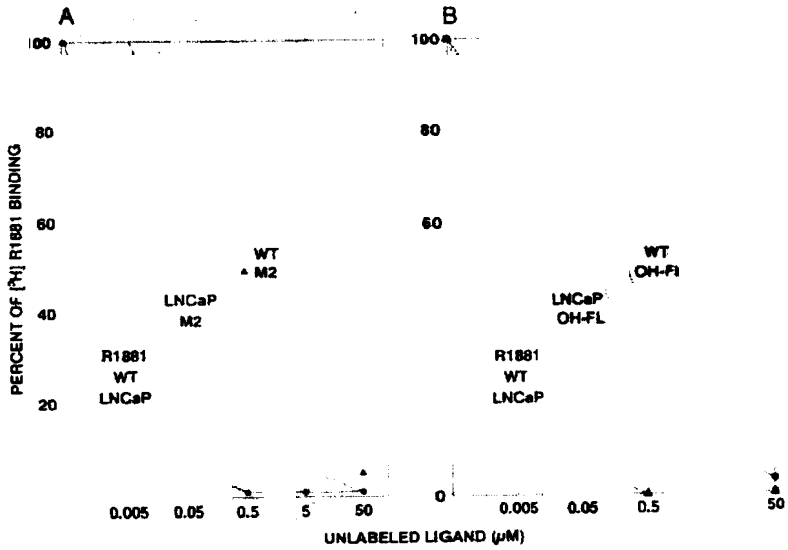

FIG. 8. **Competitive inhibition of [^{3}H]R1881 binding to wild-type and LNCaP AR by M2 and hydroxyflutamide.** Whole cell binding assays were performed in transiently transfected COS cells as described under "Experimental Procedures" and the legend of Fig. 2 using wild-type (*WT*) and LNCaP prostate cancer pCMVhAR expression vector DNA and incubating with 5 nM [^{3}H]R1881 and increasing concentrations of unlabeled R1881, M2, and hydroxyflutamide (*OH-FL*) as competitor. The data are expressed as percent of total binding observed in the presence of 5 nM [^{3}H]R1881 alone and are representative of three independent experiments.

results reveal the striking ability of the androgen antagonists, M2 and hydroxyflutamide, to act as AR agonists at high concentrations in the absence of DHT.

Relative ligand binding affinity, androgen response element DNA binding, and agonist activity of M2 and hydroxyflutamide were investigated further using an AR mutant that codes for the same amino acid sequence as the AR mutation in the human LNCaP human prostate cancer cell line. In these cells, a single base mutation within the AR gene region coding for the steroid binding domain changes threonine 877 to alanine and increases AR binding affinity for and agonist activity of hydroxyflutamide (16–18). M2 was two to three times more effective as a competitive inhibitor of [^{3}H]R1881 binding to LNCaP AR compared with wild-type AR, a binding difference similar to that observed with hydroxyflutamide (Fig. 8). Similarly, DNA binding of baculovirus expressed LNCaP mutant AR was induced by concentrations of M2 10-fold lower than required for DNA binding of wild-type AR (Fig. 9, *lane 3*). Hydroxyflutamide induced DNA binding of the LNCaP mutant AR at a 50-fold lower concentration than required for wild-type AR (Fig. 9, *lanes 5–7*). The extent of expression of wild-type and LNCaP recombinant AR was similar based on immunoblot analysis (data not shown). Similarly, the transcriptional response to M2 with LNCaP AR was 9-fold greater than with wild-type AR (Fig. 10). Transcriptional activity induced by 1 nM DHT was 44- and 68-fold with the wild-type and mutant receptors, respectively (Fig. 10). The results suggest that M2 and hydroxyflutamide can act as agonists at high concentrations (10 μM) in the absence of androgen both in cells expressing wild-type AR and at lower concentrations (0.2–1 μM) in cells expressing mutant ARs with ligand binding specificity of the threonine 877 to alanine mutation.

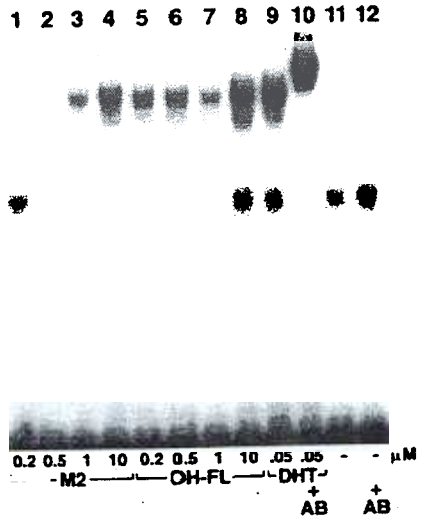

FIG. 9. **Increased DNA binding of baculovirus expressed LNCaP prostate cancer cell line mutant AR after incubation with M2 and hydroxyflutamide.** DNA mobility shift assays were performed as described under "Experimental Procedures." Sf9 cells expressing the mutant LNCaP AR from recombinant baculovirus were incubated with increasing concentrations of M2 (*lanes 1–4*) or hydroxyflutamide (*OH-FL, lanes 5–8*) between 0.2 and 10 μM or with (*lanes 9–10*) or without (*lanes 11–12*) 50 nM DHT. The *upper band* represents specific AR-DNA binding, since it is shifted to a slower migration by the addition of AR52 IgG antibody (*AB, lane 10*) and is undetected in the absence of DHT but presence of AR52 antibody (*lane 12*). The middle band represents nonspecific DNA binding by Sf9 cell extracts, and the band at the bottom of the gel is the *upper portion* of the free labeled oligonucleotide. Approximately 15,000 cpm were applied to each lane. The data shown are representative of three independent experiments.

DISCUSSION

Metabolites M1 and M2 of the fungicide vinclozolin are potential antiandrogens that inhibit AR-mediated transcriptional activity in a concentration-dependent manner by blocking androgen-induced AR binding to androgen response element DNA. M2 has a potency similar to a structurally related antiandrogen, hydroxyflutamide, whereas the major metabolite M1 is less active but could contribute to antiandrogenic potency because of its higher concentration; M1 reaches 40–75 times higher concentrations than M2 in rats administered vinclozolin. An unexpected result was the agonist activity of M2 and hydroxyflutamide in the absence of androgen. These ligands at high concentrations apparently induce a receptor conformation compatible with AR DNA binding and transcriptional activation. With the LNCaP cell mutant AR (Thr^{877} to Ala), hydroxyflutamide and M2 had agonist activity at lower concentrations due to increased binding affinity of the mutant AR for these compounds.

The results raise the possibility that mixed ligand dimers, *i.e.* agonist (natural androgens) and antagonist bound in the same dimer, are required for antagonism, whereas same ligand dimers of sufficiently high affinity promote receptor activation. A similar hypothesis was suggested for type II antagonists of PR (19). Based on trypsin digestion patterns, antihormones are

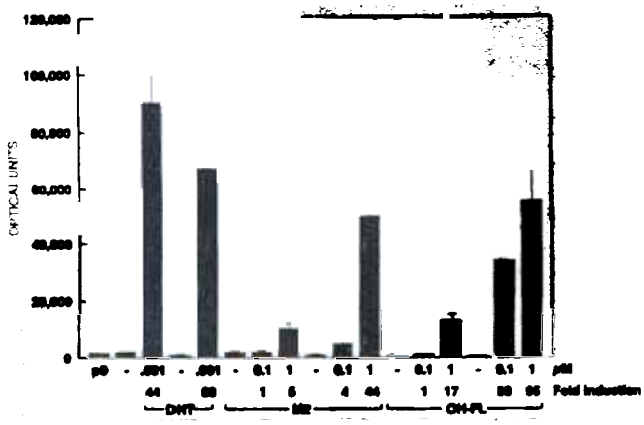

FIG. 10. **Increased agonist activity of M2 and hydroxyflutamide with the LNCaP prostate cancer cell line mutant AR.** Wild-type (*WT*) and the LNCaP mutant AR (Thr^{877} to Ala) expression vector DNAs were transiently expressed into CV1 cells with the luciferase reporter vector as described under "Experimental Procedures" and incubated with 0.1 and 1 μM M2 and hydroxyflutamide (*OH-FL*). Optical units are compared with activity determined in the presence of 1 nM DHT. -Fold induction was determined relative to the activity determined in the absence of added ligand and is indicated numerically at the *bottom*.

believed to induce inappropriate receptor conformations of PR (20) but not AR (21). Mixed ligand dimers may be transcriptionally inactive because the two ligands induce incompatible conformational states, each of which could be active in same ligand dimers. In support of distinct ligand-induced receptor conformations, steroid binding domain mutations altered the antagonist/agonist relationship in PR (22) and ER (23). Antagonist and agonist activities appear to be determined by receptor binding affinity, ligand concentration, and the presence or absence of competing high affinity natural liganda. Tissue-specific differences in ligand metabolism would then contribute to the antagonist or agonist activity of a particular compound. Differences may also exist among natural enhancers and/or promoters of androgen regulated genes.

It remains to be established the extent to which the general public and occupational workers are exposed to the widely used fungicide, vinclozolin, or whether active metabolites reach concentrations sufficient for antiandrogenic or androgenic activity. Daily oral dosing of rats results in serum M2 levels sufficient for antagonist but not agonist activity. On the other hand, flutamide administered in high doses to prostate cancer patients may be detrimental due to the agonist potential of the active metabolite, hydroxyflutamide. Plasma levels of hydroxyflutamide can reach 78 ng/ml (8 μM) (24) which is within the agonist range. Prostate cancer patients can experience improvement upon discontinuing flutamide treatment, a phenomenon known as flutamide withdrawal syndrome (25, 26). Similar improvement is reported in some breast cancer patients removed from tamoxifen (27). High level, long term exposure to flutamide, particularly in men undergoing androgen withdrawal therapy for prostate cancer, could cause proliferation of androgen-responsive cells. AR mutations like the prostate cancer cell line LNCaP mutant AR, reported in 6 of 24 advanced prostate cancer specimens (28), can enhance the agonist effects of certain antiandrogens like M2 and hydroxyflutamide because of increased binding affinity.

The results raise concern about the potential biological effects of excess exposure to the fungicide, vinclozolin, that could potentially influence normal male sexual differentiation and/or fertility. Furthermore, the use of high dose flutamide treatment in men with prostate cancer may be detrimental in some patients undergoing androgen withdrawal therapy. Finally, a relationship is suggested between antagonist and agonist activities based on the possible formation of mixed ligand or same ligand dimers, respectively.

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Vinclozolin, a widely used fungizide, enhanced BaP-induced micronucleus formation in human derived hepatoma cells by increasing CYP1A1 expression

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Abstract

Vinclozolin, a widely used fungicide, can be identified as a residue in numerous vegetable and fruit samples. To get insight in its genetic toxicity, we investigated the genotoxic effect of vinclozolin in the human derived hepatoma cell line HepG2 using the micronucleus (MN) assay. Additionally, to evaluate the co- or anti-mutagenic potency of vinclozolin, we treated HepG2 cells with different concentrations of vinclozolin for 24 h. Subsequently, the cells were exposed to benzo[*a*]pyrene (BaP) for 1 h. Exposure of HepG2 cells to 50–400 μM vinclozolin alone did not cause any induction of micronuclei. However, a pronounced co-mutagenic effect was observed. MN frequencies caused by BaP increased by 30.6%, 52.8% and 65.3% after pretreatment of the cell cultures with 50, 100 and 200 μM vinclozolin, respectively. The highest concentration (400 μM) of vinclozolin tested caused cytotoxicity. Therefore, micronuclei were not considered for that concentration. To clarify the mechanism of cogenotoxicity, we assayed cytochrome P450 1A1 (CYP1A1), which plays a pivotal role in activation of BaP. Cells exposed to vinclozolin led to significant increase of CYP1A1 expression in Western blot. The result suggested that induction of CYP1A1 by vinclozolin account for its enhancing effect on genotoxicity caused by BaP.

Keywords: Vinclozolin; HepG2 cells; Co-mutagenicity; Micronucleus assay YP1A

Introduction

3-(3,5-Dichlorophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedione (vinclozolin) is used in agriculture over a period of more than 20 years to protect fruits and

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vegetables from various fungal pathogens (Davidek et al., 1981). Since vinclozolin can be detected in many plant foodstuffs in low concentrations as a residue and farmers may be exposed to the fungicide during preparation and application, the human risk should be well evaluated. In the last two decades, most papers on vinclozolin focused on its anti-androgenic effects (Kelce et al., 1994; Hoyer, 2001). However, some studies dealing with the genetic toxicity of vinclozolin and vinclozolin mediated lipid peroxidation were also published (Lioi et al., 1998).

So far, results reported on mutagenicity and carcinogenicity of vinclozolin are various. Several studies from U.S.E.P.A. including the *Salmonella* assay, a Chinese hamster sister chromatid exchange assay, a host-mediated assay with *Salmonella typhimurium*, and a dominant lethal test in mice showed that vinclozolin is a non-genotoxicant (EPA, 1985). Additionally, it did not induce any significant increase of chromosomal aberrations in human peripheral blood lymphocytes in the presence and absence of S9 (Hrelia et al., 1996) and it did not show increased sister-chromatid exchanges (SCE) in human lymphocytes in vitro (±S9) (Kevekordes et al., 1996). The rats orally dosed with Vinclozolin did not lead to the formation of haemoglobin adducts (Sabbioni and Neumann, 1990). Likewise, no significant micronucleated polychromatic erythrocytes in bone marrow was found after the mice orally exposed to vinclozolin indicating that vinclozolin is neither clastogenic nor aneugenic (Kevekordes et al., 1996). Although most studies insisted on non-genotoxicity of vinclozolin, a few studies showed conflicting results. In studies of Hrelia et al. (1996), vinclozolin induced micronuclei in erythrocytes in the bone marrow of male mice. Perocco reported cell transformation in BALB/c 3T3 cells exposed to vinclozolin (±S9) (Perocco et al., 1993). Additionally, in contrast to former studies, Lioi found a statistically significant increase of structural aberrations and SCE in bovine lymphocyte cultures in vitro induced by vinclozolin (Lioi et al., 1998). A review from U.S.E.P.A. showed that vinclozolin induced Leukaemia and lymphoma in male animals and also some adenoma in the lung of female mice (EPA, 1985). Overall, present knowledge based on majority of negative outcomes tends to suggest that vinclozolin is not a direct DNA damaging agent.

In a former study, Hrelia et al. (1996) and Ronis et al. (1994) demonstrated that vinclozolin can increase the expression of various cytochrome P450-dependent oxigenases (CYPs). It is recognized that CYPs are key factors in the activation of progenotoxicants to its ultimate genotoxic metabolites. Therefore, in present study, we introduced a human derived metabolically competent cell line (HepG2) to study genotoxicity. HepG2 cells retained the activity of most of the xenobiotic metabolizing enzymes (Grant et al., 1988; Doostdar et al., 1993; Chung and Bresnick, 1994; Kress and Greenlee, 1997; Krusekopf et al., 1997; Delescluse et al., 1998). Additionally, several studies demonstrated the ability of HepG2 cells to activate different types of progenotoxicants (Natarajan and Darroudi, 1991; Knasmuller et al., 1998). Our group demonstrated the suitability of HepG2 cells to detect cogenotoxicants by identifying the synergism of musk ketone and BaP (Mersch-Sundermann et al., 2001). Because of these advantages, the present study will provide some information regarding the elucidation of genotoxicity of vinclozolin and its role in the modulation of the activity of progenotoxicants.

2. Materials and methods

2.1. Chemicals

BaP (CAS 50-32-8) and vinclozolin (CAS 50471-44-8) were purchased from Sigma (St. Louis, MO, USA). Vinclozolin and BaP were diluted in filter sterilized DMSO (Sigma) with final concentration in the medium of less than 1%. Chemicals related to enzyme measurement were purchased from Sigma. All other chemicals and solvents were of the highest purity commercially available (analytical grade). Dulbecco's minimal essential medium (DMEM), fetal calf serum and antibiotics (penicillin, 100 U/ml, streptomycin 0.1 mg/ml) were obtained from Gibco Company (Paisley, UK).

2.2. Cells and culture conditions

The HepG2 cell line was originally established from a human liver tumor biopsy and kindly provided by Dr. Firouz Darroudi (Leiden, Netherlands). The cells were grown in DMEM supplemented with 15%

fetal calf serum, penicillin and streptomycin at (5% CO_2).

2.3. Micronucleus/HepG2 assay

The assays were performed as described by Natarajan and Darroudi (1991), partly been modified. Briefly, 10^6 cells were grown in culture flasks (25 cm^2) containing 5 ml of complete medium (24 h). Then, the cultures were treated with various concentrations of vinclozolin (24 h). Subsequently, the cells were washed twice with PBS and incubated in complete growth medium supplemented with 3 μg/ml cytochalasin B1 (24 h). At last, the cells were trypsinized and treated with cold hypotonic KCl (5.6 g/l). Then, the cells were centrifuged and an acetic acid–methanol (1:3) solution and three drops of formaldehyde were gently added. The fixation step was repeated three times and the resulting cells were re-suspended in a small volume of fixative solution. Air-dried preparations were made and slides were stained with 2.5% Giemsa (Gurr66) solution. For each concentration of the test compound 1000 binucleated cells (BNC) were evaluated for micronuclei (MN). The data are presented as the number of MN per 1000 BNC. MN were scored according to established criteria (Kirsch-Volders et al., 2000) with a Leica microscope. To rule out cytotoxic effects, the nuclear division index (NDI) of each sample was evaluated according to (Eastmond and Tucker, 1989). Three independent experiments were carried out for each culture set ($n=3$).

The protocol of the cogenotoxicity assay was from (Mersch-Sundermann et al., 2001). Namely, the cells were pretreated with vinclozolin for 24 h, washed twice with PBS and thereafter cultivated in medium, which contained 50 μM BaP (50 μM) for 1 h. Then, the procedure was identical with that described above.

2.4. Western blotting

To determine the CYP1A1 expression HepG2 cells were treated with 200 and 400 μM vinclozolin (24 h). Trypsin was used to collect cells prior to washing with PBS. The cell pellets were resuspended in lysis buffer containing 10 mM Tris–HCl (pH 7.4), 5 mM EDTA, 150 mM NaCl, 0.5% IGEPAL CA-630 (Sigma). The cytosolic protein concentration was determined by Bradford assay (Bradford, 1976) with bovine serum albumin as the standard. Cell lysates, containing equal amounts of protein, were boiled in SDS-sample buffer for 5 min before loading on a SDS polyacrylamide gel. Proteins were transferred to nitrocellular membranes (Amersham, Freiburg, Germany). Then, the membranes were blocked with 5% non-fat dry milk in TBS-T pH 8.0 and incubated with 1:300 monoclonal antibodies against CYP1A1 (CYP2000, Bochum, Germany) overnight at 4°. After washing three times with TBS/T, the HRP-linked secondary antibodies (Cell signaling, Beverly, MA) were added and incubated for 1 h at room temperature. At last, the membranes were washed with TBS/T for three times and the immunoreactive bands were visualized using ECL chemiluminescence detection agents (Amersham, Freiburg, Germany). Tow independent tests were performed.

2.5. Statistical evaluation

Statistical significance was evaluated by Dunnet's *t*-test. Statistical significance was accepted within $p \leq 0.05$.

3. Results

Prior to the experiments, pilot tests were performed to determine the appropriate test doses and conditions for vinclozolin and BaP. The concentration (200 μM for 24 h incubation, percentage of binucleated cells is over 50%) in the pilot study was chosen as the highest vinclozolin dose in the experiments. For BaP we tracked down a concentration located in the linear region of the dose/MN curve (50 μM for 1 h incubation), which was capable of recognizing both up regulation and down regulation of the MN frequency (Fig. 1).

In the first assay, we investigated the effect of vinclozolin alone on the MN frequency in HepG2. As shown in Table 1 vinclozolin slight increase of MN frequency. In particular, the highest concentration induced a MN frequency of 35. However, the low nuclear division index suggests the concentration induced strong cytotoxicity. The slight increase induced by the other concentrations of vinclozolin is not statistically significant.

In the combination assay, we studied the effects of vinclozolin on the genotoxicity of BaP. The 50 μM BaP reference control (pretreated only with DMSO) caused a significant, over two-fold increase of MN over